New insights into the structure and mechanism of iodothyronine deiodinases

Ulrich Schweizer and **Clemens Steegborn[1]**

Institut für Biochemie und Molekularbiologie, Rheinische Friedrich-Wilhelms-Universität Bonn, Nussallee 11, 53115 Bonn, Germany
[1]Lehrstuhl Biochemie, Universität Bayreuth, Universitätsstrasse 30, 95445 Bayreuth, Germany

Correspondence should be addressed to U Schweizer
Email ulrich.schweizer@uni-bonn.de

Abstract

Iodothyronine deiodinases are a family of enzymes that remove specific iodine atoms from one of the two aromatic rings in thyroid hormones (THs). They thereby fine-tune local TH concentrations and cellular TH signaling. Deiodinases catalyze a remarkable biochemical reaction, i.e., the reductive elimination of a halogenide from an aromatic ring. In metazoans, deiodinases depend on the rare amino acid selenocysteine. The recent solution of the first experimental structure of a deiodinase catalytic domain allowed for a reappraisal of the many mechanistic and mutagenesis data that had been accumulated over more than 30 years. Hence, the structure generates new impetus for research directed at understanding catalytic mechanism, substrate specificity, and regulation of deiodinases. This review will focus on structural and mechanistic aspects of iodothyronine deiodinases and briefly compare these enzymes with dehalogenases, which catalyze related reactions. A general mechanism for the selenium-dependent deiodinase reaction will be described, which integrates the mouse deiodinase 3 crystal structure and biochemical studies. We will summarize further, sometimes isoform-specific molecular features of deiodinase catalysis and regulation, and we will then discuss available compounds for modulating deiodinase activity for therapeutic purposes.

Introduction

Thyroid hormones (THs) are essential for developmental processes, growth, and the regulation of energy metabolism (Tata 1968, Bianco & Kim 2006, Mullur *et al*. 2014). In the thyroid gland, tyrosine residues within thyroglobulin are enzymatically iodinated yielding mono- and diiodotyrosine moieties, some of which are further coupled via an ether linkage to yield iodothyronines (Salvatore *et al*. 2011). Iodotyrosines and TH are liberated from thyroglobulin by proteases (Friedrichs *et al*. 2003). Specific monodeiodination reactions are responsible for both TH activation and inactivation in target tissues

(Schweizer *et al*. 2008). The principal hormone activating the nuclear TH receptor is 3,3′,5-triiodothyronine (T_3). It is generated from the main secreted product of the thyroid gland, thyroxine (T_4, 3,3′,5,5′-tetraiodothyronine), by elimination (deiodination) of the 5′-iodine atom from the outer iodothyronine ring (Fig. 1). Removal of an additional iodine atom from the inner or the outer ring results in 3,3′-diiodothyronine (3,3′-T_2) or 3,5-T_2 respectively. While the former metabolite is believed to be inactive, the latter is increasingly recognized as a TH-receptor activating ligand in fish and, at higher
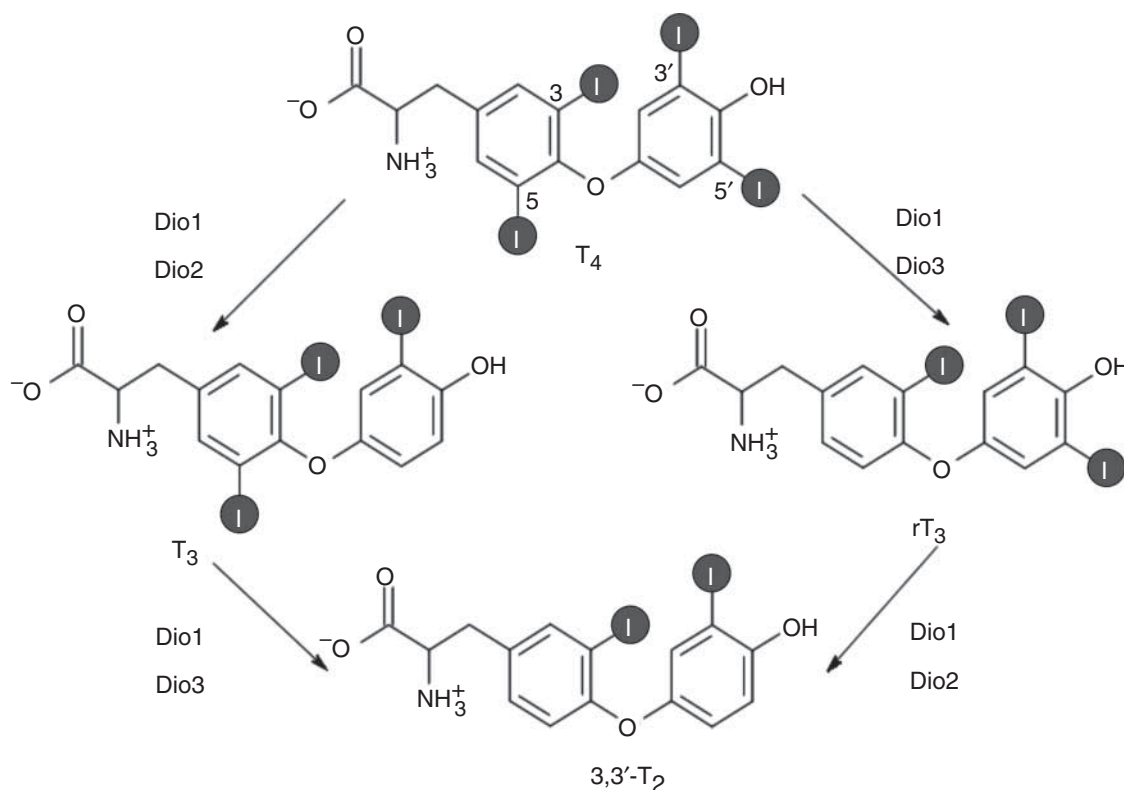
Figure 1
Reactions catalyzed by mammalian iodothyronine deiodinases.

concentrations, in mice (Mendoza *et al.* 2013, Orozco *et al.* 2014, Jonas *et al.* 2015). T_4 can also be directly inactivated by the removal of an inner ring (5-)iodine resulting in reverse T_3 (rT_3). In mammals, the different deiodination reactions are catalyzed by three different deiodinase enzymes (Dio1–3), which specifically target either the inner or outer ring or both of them (Fig. 1A). Cell type-specific and developmental expression patterns of deiodinase isoenzymes can thus fine-tune systemic or local levels of TH and adapt T_3 levels to physiological situations, including tissue injury, regeneration and carcinogenesis (Dentice *et al.* 2013a,b, Mullur *et al.* 2014).

Many facets of TH signaling are well understood, revealing a nicely orchestrated interplay of different tissues and molecular players (Bianco & Kim 2006, Mullur *et al.* 2014). Deiodinases have well described key functions in this system, but their molecular mechanisms of catalysis and regulation are not fully understood despite many studies on these topics. The lack of molecular insight likely contributes to the paucity of small molecule modulators for deiodinases, which could be potential drugs for the treatment of some forms of hyperthyroidism, hypothyroidism, and cancer (Manna *et al.* 2013,

Ciavardelli *et al.* 2014). We will review the reaction mechanism, regulation and molecular structure of deiodinases based on the recently determined crystal structure of mouse Dio3 (mDio3; Schweizer *et al.* 2014a). We will shortly discuss modulatory compounds and emerging drug development opportunities.

There are many ways to skin a cat: dehalogenating and deiodinating enzymes

Elimination of halogen atoms from aromatic rings is a demanding type of reaction under physiological conditions. Yet, nature has devised structurally different enzymes following different catalytic mechanisms to complete this task. Dehalogenation of iodotyrosine was reported in thyroid extract as early as 1950 (Hartmann 1950). 3-Monoiodotyrosine and 3,5-diiodotyrosine are major degradation products of thyroglobulin that have not been incorporated into THs. Lack of iodotyrosine deiodinase (IYD) activity in patients, if left untreated, leads to clinical hypothyroidism and mental retardation (Moreno & Visser 2010). The gene encoding IYD has been cloned and renamed dehalogenase (DEHAL1;

Published by Bioscientifica Ltd.

Moreno 2003, Gnidehou *et al.* 2004). Patients carrying mutations in DEHAL1 have been identified and suffer from TH deficiency (Moreno *et al.* 2008). IYD is a flavoenzyme and uses NADPH for reductive deiodination (Goswami & Rosenberg 1977, McTamney & Rokita 2009). It is a membrane-associated enzyme and its crystal structure was solved after establishing efficient recombinant expression (Thomas *et al.* 2009). The NADPH-dependence of IYD was lost upon solubilization, however, a finding that has been explained by loss of an unidentified intermediate electron carrier connecting NADPH and IYD (Rokita *et al.* 2010). The exact mechanism of IYD is not established, but it is clear that it does not involve a catalytic thiol and relies instead on the presence of a protein-bound FMN (Hu *et al.* 2015; Fig. 2A).

The following paragraph provides a summary of the many different mechanisms employed for aromatic dehalogenation by non-flavoenzymes. While the casual reader may skip the paragraph, we feel that a comparison of these mechanisms is valuable to understand the deiodinase mechanism in a broader context. In contrast to vertebrate dehalogenase, reductive dehalogenases from organohalide-respiring bacteria require an anaerobic environment to break down polychlorinated biphenyls or other environmental pollutants. In these bacterial dehalogenases, the cobalt atom from a cobalamin cofactor interacts with, and provides electrons to the halogen during elimination (Fig. 2B; Bommer *et al.* 2014, Payne *et al.* 2015). Two iron–sulfur clusters nearby are probably involved in catalysis and subsequent reduction of the cobalt atom. Two potential mechanisms were discussed by Payne *et al.* (2015): one involving keto–enol tautomerism with a vicinal phenol, and one involving homolytic cleavage of the halide–carbon bond after an electron transfer and carbanion formation (Fig. 2B). Another bacterial cobalamin-dependent dehalogenase does not depend on the presence of a vicinal hydroxyl group, suggesting that the mechanism without keto intermediate is likely employed by this type of enzyme (Bommer *et al.* 2014). Bacterial tetrachlorohydroquinone dehalogenase (TCHQ), a distant member of the glutathione-*S*-transferase gene family, is a glutathione-dependent dehalogenating enzyme. TCHQ catalysis is thought to involve a keto–enol tautomerism followed by nucleophilic substitution of the thereby activated chloride by glutathione and subsequent resolution of the covalent thioether intermediate (Crooks *et al.* 2010; Fig. 2C). Keto–enol tautomerism and a covalent intermediate has also been suggested for the extensively studied dehalogenation reaction catalyzed by thymidylate synthase acting on 5-iodo-uridine (Carreras & Santi 1995).

Here, the halogen atom is first activated by attaching an enzyme thiolate in ortho-position, followed by an attack of another, reducing thiol on the halide (Fig. 2D). Yet another dehalogenation mechanism has been proposed for deiodinases based on work with synthetic small molecule mimics with outer ring deiodinase activity (Goto *et al.* 2010): keto–enol tautomerism of the deiodination position and the phenol group in ortho activates the iodine, which is then attacked by a selenol (Fig. 2E). Resolution of the ketone eliminates a selenenyliodide. However, the reaction conditions in chloroform as solvent, at 50 °C, and over a period of 1 week were far from physiological. Also, ketone formation is not possible for inner ring deiodination. We favor the idea of a common mechanism for both types of thyronine deiodination, excluding mechanisms with keto intermediate (see below). An aromatic substitution via an addition/elimination mechanism with the selenol as nucleophile (Fig. 2F) has been discussed based on work with another synthetic deiodinase mimic that is capable of inner ring deiodination at physiological buffer and temperature conditions (Manna & Mugesh 2010). These authors favored, however, yet another mechanism based on halogen bond formation between the selenol and the aromatic iodine substituent, followed by selenenyliodide release and reprotonation of the resulting aromatic carbanion (Fig. 2G; Manna & Mugesh 2010, 2012).

Mammalian deiodinase enzymes do not bind flavin or corrin co-factors, but share a conserved and essential Sec residue. Exchange of Sec for Cys reduces activity by a factor of 100 (Berry *et al.* 1992, Kuiper *et al.* 2003*a*), and this residue thus appears to play the key role in deiodinase catalysis via one of the thiol/selenol-based mechanisms discussed above. While Dio2 catalyzes outer ring (5′-)deiodination, which could potentially follow one of the keto–enol mechanisms, the closely related isoforms Dio1 and Dio3 (see below) are capable of inner ring (5-)deiodination, a reaction that cannot involve a keto-intermediate of the substrate. Since formation of a selenenyliodide intermediate is widely accepted (Kuiper *et al.* 2005*a*), the aromatic substitution mechanism appears less likely and the widely favored mechanism seems to be the iodonium elimination by a selenolate via halogen bond formation, selenyliodide release and reprotonation of an aromatic carbanion (Fig. 2G). The Se–halide bond would be analogous to the Co–halide bond in bacterial dehalogenase, i.e., the selenium (or sulfur) would provide the electron pair required for the two-electron reduction. Oxidized deiodinase is then regenerated by thiols – DTT (*in vitro*), glutathione, or protein thiols.
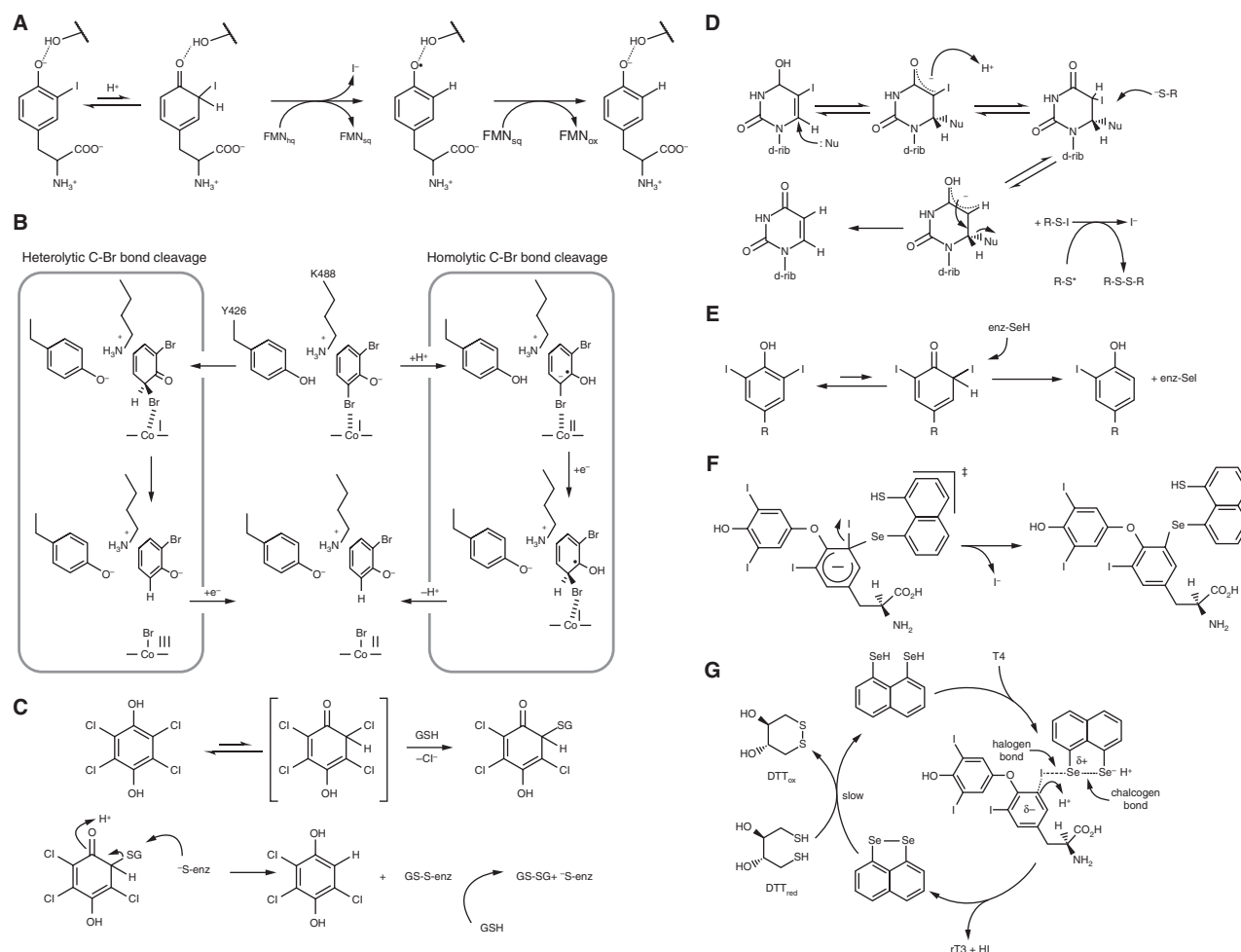
Figure 2

Different dehalogenation mechanisms. (A) Flavin-dependent iodotyrosine dehalogenase (IYD; reproduced, with permission, from Hu J, Chuenchor W & Rokita SE 2015 A switch between one- and two-electron chemistry of the human flavoprotein iodotyrosine deiodinase is controlled by substrate. *Journal of Biological Chemistry* **290** 590–600 (copyright 2015, American Society for Biochemistry and Molecular Biology)). (B) Bacterial cobalamin-dependent dehalogenase (reproduced, with permission, from Macmillan Publishers Ltd: Payne KA, Quezada CP, Fisher K, Dunstan MS, Collins FA, Sjuts H, Levy C, Hay S, Rigby SE & Leys D 2015 Reductive dehalogenase structure suggests a mechanism for B12-dependent dehalogenation. *Nature* **517** 513–516 (copyright 2015)). (C) Bacterial glutathione-dependent tetrachlorohydroquinone dehalogenase (TCHQ; data from Rokita *et al*. (2010)). (D) Deiodination of 5-iodo-uridine by thymidylate synthase. d-rib, deoxyribose (data from Carreras & Santi (1995)). (E) Small compound selenol-dependent 5′-deiodination of thyroxine via keto intermediate (data from Goto *et al*. (2010)). (F) Aromatic addition/elimination mechanism during 5-deiodination of thyroxine (reproduced, with permission, from Manna D & Mugesh G 2012 Regioselective deiodination of thyroxine by iodothyronine deiodinase mimics: an unusual mechanistic pathway involving cooperative chalcogen and halogen bonding. *Journal of the American Chemical Society* **134** 4269–4279 (copyright 2012, American Chemical Society)). (G) Small molecule deiodinase mimic catalyzing 5-deiodination without the need of a keto intermediate (reproduced, with permission, from Manna D & Mugesh G 2012 Regioselective deiodination of thyroxine by iodothyronine deiodinase mimics: an unusual mechanistic pathway involving cooperative chalcogen and halogen bonding. *Journal of the American Chemical Society* **134** 4269–4279 (copyright 2012, American Chemical Society)).

It should be clear from this account that the exact biochemical mechanism of mammalian deiodinases is far from resolved. In order to gain better insight into the deiodinase mechanism, we thus solved a crystal structure of the catalytic domain of mDio3 (Schweizer *et al*. 2014*a*) and used it for reevaluating previous biochemical results and suggested catalytic mechanisms.

The deiodinase catalytic domain structure made crystal clear

Although there are differences between the three mammalian deiodinases concerning their region-specificities, they all form an evolutionary-related family-sharing significant sequence homology and most architectural

and catalytic properties. The biophysical and structural characterization of deiodinases is severely aggravated by the fact that they are integral transmembrane proteins and selenoenzymes. The mechanism of Sec incorporation differs between eukaryotes and bacteria, hampering the expression of fully active, recombinant mammalian deiodinase protein in the widely used, efficient prokaryotic expression systems (Kuiper *et al.* 2005b). Expression of a Cys-mutant Dio1 in yeast yielded an active but heterogenous protein that did not allow structural studies (Kuiper *et al.* 2005b). In the absence of experimental structural information, modeling of human deiodinases suggested that a Dio-specific insertion in the Trx-like sequence forms a glycoside hydrolase like insertion in the basic Trx fold (Callebaut *et al.* 2003). The insertion was predicted to form a helix embedded in a loop and to provide the docking site for the thyronine substrate (Callebaut *et al.* 2003). Site-directed mutagenesis further revealed several essential amino acids (Callebaut *et al.*

2003) whose function, however, could not be rationalized from this model.

Only recently, we succeeded in establishing the efficient recombinant expression of an isolated mammalian deiodinase catalytic domain for structural studies (Schweizer *et al.* 2014a). In this deiodinase construct, the N-terminal region responsible for membrane integration – and possibly cellular localization (Olvera *et al.* 2015) – and the linker connecting it to the catalytic core have been omitted. Furthermore, the active site selenocysteine was replaced by a less active cysteine, which is more efficiently inserted during translation (Schweizer *et al.* 2014a). Crystal structure analysis of this inactive mDio3 catalytic domain (mDio3^{cat}) allowed us to settle the basic architecture of this enzyme family and to define the arrangement of key active site residues (Callebaut *et al.* 2003, Schweizer *et al.* 2014a). Both features suggested several conclusions relevant for the deiodinase catalytic mechanism. The structure of the apoenzyme (Fig. 3A and B),
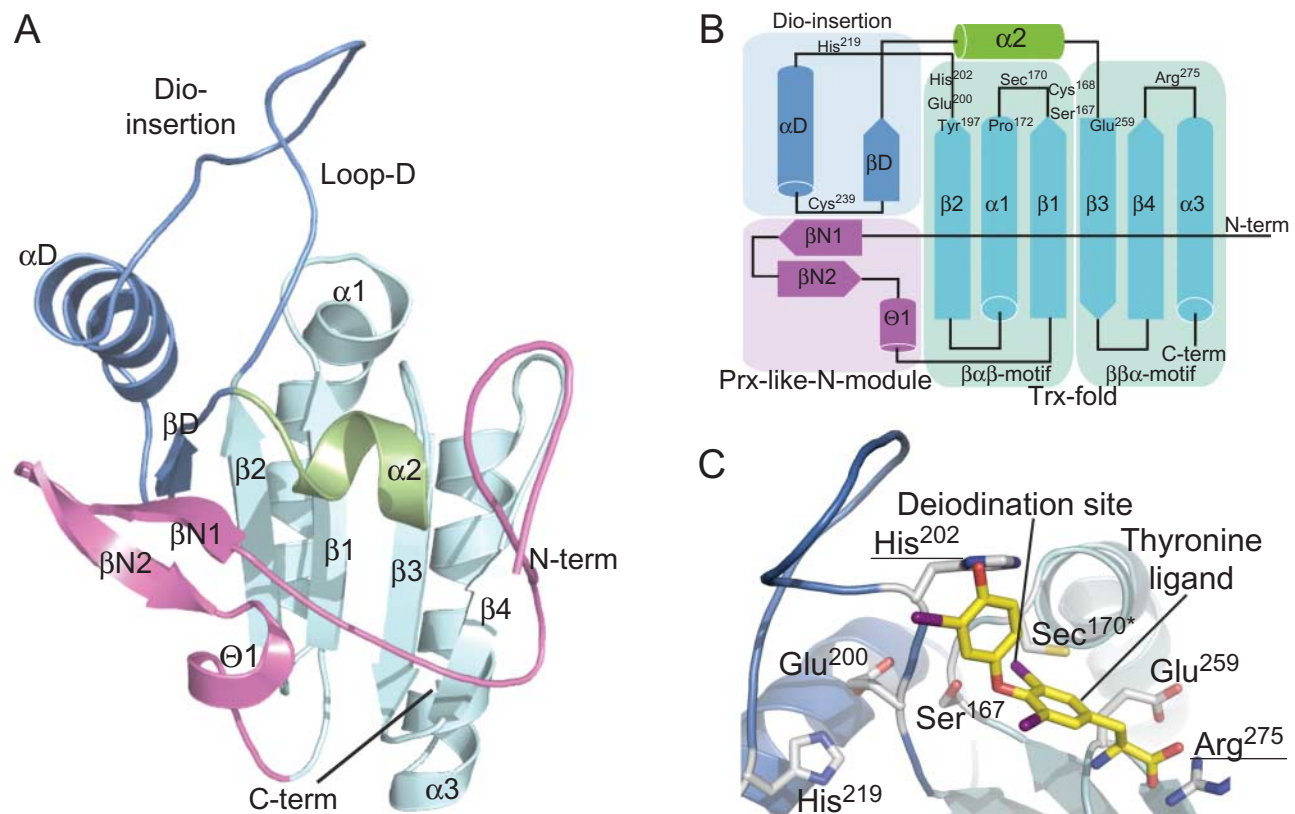

Figure 3

Structure of mammalian deiodinase catalytic domain. (A) Crystal structure of mDio3^{cat}. (B) Scheme of the mDio3^{cat} topology. (C) mDio3^{cat} active site with modeled thyronine ligand. Reproduced, with permission, from Schweizer U, Schlicker C, Braun D, Köhrle J & Steegborn C 2014a The crystal structure of mammalian selenocysteine-dependent iodothyronine deiodinase suggests a peroxiredoxin-like catalytic mechanism. *PNAS* **111** 10526–10531.

solved at 1.9 Å resolution, confirmed the general relationship to Trx-fold proteins as predicted based on sequence homologies (Callebaut *et al.* 2003, Schweizer *et al.* 2014a). N-terminal to a generic Trx βαβ-motif, however, mDio3^{cat} features an unexpected two-stranded β-sheet, βN, followed by a 3_{10}-helix Θ_1. This arrangement is typical for a peroxiredoxin (Prx) family within the Trx-fold superfamily and together with additional Prx-like features suggests a mechanistic relationship to Prx (see below). The structure further revealed that the deiodinase-specific insertion in the generic Trx fold sequence ('Dio-insertion' in Fig. 3A and B) forms a loop D, a helix αD, and a short strand βD that aligns with the central β-sheet.

The Cys replacing the catalytic mDio3 Sec^{170} (Cys^{170}*) is located between β1 and α1, corresponding to the position of the peroxidatic cysteine of Trx-fold thiol reductases (Trx, thioredoxin peroxidases, Prx, and glutathione peroxidases (Gpx)). The Sec residue points into an extended cleft, which appears to act as a substrate binding site (Fig. 3C). mDio3-His^{219}, which corresponds to the conserved and catalytically important Dio1-His^{174}, is oriented into the domain core and interacts with the conserved Glu^{200} (see below). Dio3-His^{202}, corresponding to the catalytically essential Dio1-His^{158} of the Dio-insertion, forms the end of this substrate binding cleft (Fig. 3C). It likely binds the substrate 4′-phenol, similar to His^{435} in T$_3$-receptor B (TRβ; Nascimento *et al.* 2006). A first model for a Dio substrate complex could thus be generated by superimposing the T$_3$/TRβ complex His^{435}–T$_3$–Arg^{282} clamp on Dio3-His^{202}–Arg^{275} (Fig. 3C). The 5-iodine would be ∼3–4 Å separated from the Sec^{170} selenium, consistent with the proposed selenenyliodide formation during catalysis (Bayse & Rafferty 2010; see below). In agreement with this binding mode, activity is dramatically reduced in a mutant mDio3 with Arg^{275} replaced by Ala (Schweizer *et al.* 2014a). In the thyronine complex model, Dio3-Glu^{259} appears well positioned for recognizing the substrate amino group. Mutation of the corresponding Dio1-Glu^{214} consistently increased the thyronine K$_M$ (Callebaut *et al.* 2003).

A model of deiodinase catalysis

The selenol   Selenols are thought to exist mainly in the ionic, selenolate form under physiological conditions. Thus, they are superior nucleophiles as compared to thiols, which require nearby proton acceptors and sometimes nearby positive charges for their activation. No proton acceptors or positive charges are apparent in the mDio3^{cat} structure near the Sec (Schweizer *et al.* 2014a),

consistent with the dramatic loss of deiodinase activity when mutating the active site Sec to Cys (Berry *et al.* 1992, Kuiper *et al.* 2003a). The requirement of an active site Sec in deiodinase might reflect that its substrate, and thus the substrate binding site, is much more hydrophobic than substrates for other Prx family members.

Different mechanisms have been proposed for mammalian iodothyronine deiodinases (see above): the mechanism most likely involves formation of a selenenyl-iodide intermediate (Kuiper *et al.* 2005a), as it would likewise work for inner and outer ring deiodination. In contrast, keto–enol tautomerization is only possible in the outer ring (Goto *et al.* 2010, Manna *et al.* 2015). Accordingly, a keto–enol mechanism for 5′-deiodination would call for an entirely different catalytic mechanism for 5-deiodination. Owing to the high homology of the Dio1–3 enzymes, we deem this rather unlikely and favor a common selenenyliodide pathway, with a direct halogen bond attack on the aromatic iodine substituent, for all deiodinations they catalyze. Studies with small molecule mimics for deiodination support this notion of a common mechanism for inner and outer ring deiodination (Manna *et al.* 2015). Also, energetic considerations and density functional theory calculations support an in-line attack of the selenolate onto the iodine atom within the aromatic plane (Bayse & Rafferty 2010, Manna *et al.* 2015), reminiscent of the cobalt-dependent mechanism reported recently for a bacterial dehalogenase (Payne *et al.* 2015) (see also above). A model for a thyronine complex of the mDio3^{cat} domain would indeed position the substrate iodine properly for an in-line attack on the halogen (Manna & Mugesh 2010). The selenenyliodide mechanism would also be consistent with results from small molecule enzyme models and deiodinase inhibitors (Manna & Mugesh 2010, Manna *et al.* 2013, 2015; see below).

Differential sensitivity among deiodinase isoenzymes to inhibition by propylthiouracil (PTU; see below) has served as an important argument supporting two different catalytic mechanisms (Kuiper *et al.* 2005a). Our structure of mDio3^{cat} and models of Dio2 and Dio1 suggest a structural – rather than a mechanistic – reason for differential PTU-sensitivity. PTU inhibits Dio1 only in the presence of substrate, and iodide is released from Dio1 before inhibition (Kuiper *et al.* 2005a). Dio2 and Dio3, in contrast, are insensitive to PTU. The insensitivity to PTU was linked to a Pro at position +2 after the Sec (Fig. 4A). Exchange of Pro present at this position in *Xenopus* Dio1 to the Ser found in human Dio1 made the frog enzyme PTU sensitive (Kuiper *et al.* 2006). Likewise, mutation of the Pro present in human Dio2 to Ser conferred PTU sensitivity to
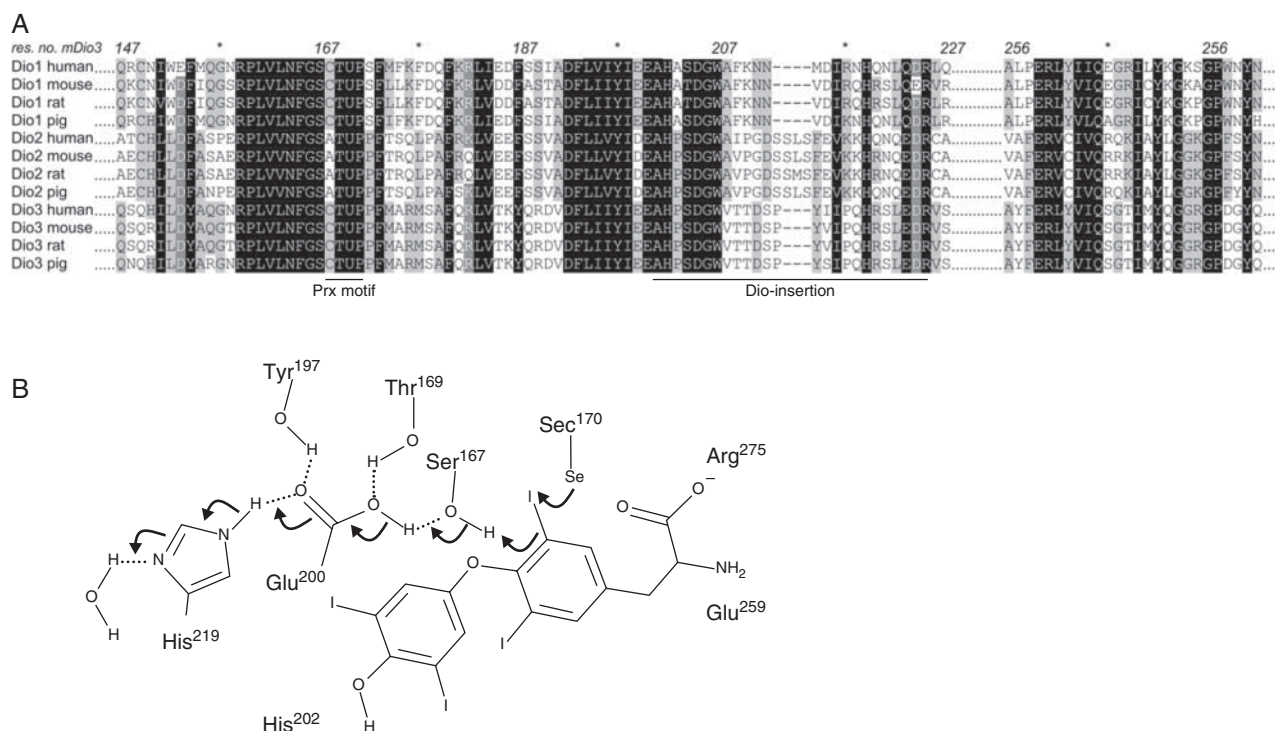
Figure 4

Conserved amino acids in the deiodinase active site. (A) Multiple alignment showing the conserved 'Prx motif' and the 'Dio insertion'. (B) Amino acids possibly involved in the donation of a proton to the deiodinated substrate.

this enzyme (Goemann *et al.* 2010). The mDio3^{cat} structure indicates that the bulkier Pro may limit accessibility to the oxidized Sec (e.g. in a selenenylsulfide) to PTU and thereby prevent inhibition. Macroscopic kinetic differences, i.e., the 'ping–pong' mechanism observed in Dio1 and sequential mechanisms observed in Dio2 and Dio3, have also been interpreted as arguments in favor of different microscopic mechanisms of deiodinases. However, the described Pro/Ser substitutions have also changed macroscopic kinetics in recombinant deiodinase enzymes (Callebaut *et al.* 2003, Goemann *et al.* 2010), suggesting that the limitation of the conformational space or the microscopic kinetic effects associated with Pro at this position determine the macroscopic mechanisms.

The proton An aspect that has not attracted much attention until recently is the source of the hydrogen or proton that ultimately replaces the iodine in the thyronine product. Assuming the selenenyliodide mechanism, a proton is required to replace the iodonium, which forms the selenenyliodide. In the mDio3^{cat} structure, we recognized a hydrogen bond network connecting the

catalytic loop with the distant, but essential Dio3-His^{219}. In our interpretation, this H-bond network could convey a proton from the solvent via His^{219}, Glu^{200}, Thr^{169}, and Ser^{167} to the iodothyronine ring (Fig. 4B). Tyr^{197}, which was previously thought to participate in dimerization, is buried deep within the mDio3^{cat} structure and helps establish the H-bond network. Donation of a proton by the essential Ser^{167}, which is part of a Prx signature motif Ser/Thr-X-X-Sec/Cys also conserved in deiodinases (Fig. 4A), is again reminiscent of a proton-donating Tyr in a Tyr-Lys/Arg dyad as in the bacterial dehalogenase (Bommer *et al.* 2014, Payne *et al.* 2015). Alternatively, the identified H-bond network might position a water molecule close to the active site as a proton source, but this model would hardly explain the catalytic importance of the more distant network residues.

The source of electrons Selenenyliodide formation and thyronine protonation results in a reduced substrate and an oxidized active site selenium. The iodide might be released through spontaneous hydrolysis (leaving an oxidized selenenic acid moiety, -SeOH) or during the re-reduction of the selenium atom, and a major

outstanding question pertaining deiodinase catalysis concerns the mechanism of this reduction and regeneration of the enzyme. Unlike bacterial co-dependent dehalogenase (Payne *et al.* 2015), deiodinases do not contain an Fe–S cluster for subsequent reduction of the catalytic atom. However, deiodinases contain one to two conserved Cys residues, which have repeatedly been proposed to participate in enzyme regeneration or interaction with reducing co-factors (Sun *et al.* 1997, Croteau *et al.* 1998, Kuiper *et al.* 2002). The Dio3-Cys168Ala mutant reduces enzymatic activity by about 50% irrespective of using DTT or protein thiols (Schweizer *et al.* 2014*a*), in accordance with earlier findings regarding Dio1-Cys126Ala (Sun *et al.* 1997, Croteau *et al.* 1998). Insertion of a corresponding Cys into Dio2, which lacks this second Cys in its native sequence, accordingly stimulates enzyme activity in the presence of DTT (Kuiper *et al.* 2002).

One problem in analyzing the deiodinase mechanism is that it has been mostly studied in the presence of DTT, a non-physiological co-substrate. Using DTT for the determination of deiodinase activity in tissue extracts is useful, e.g., if a measure for the relative amount of enzyme is sought. Estimates of TH economy and mechanistic analyses of deiodinase catalysis have also been generated based on assays employing this artificial co-substrate DTT (Maia *et al.* 2005). The meaning and limitations of such numbers has to be considered very carefully, because 20–50 mM concentrations of this non-physiological

co-substrate influence enzyme kinetics and mechanism. The identity of the physiological reducing co-substrate is still not clear, but early studies have suggested small protein thiols as physiological co-substrates (Goswami & Rosenberg 1985, Bhat *et al.* 1989). We have recently reconstituted functional regenerating systems *in vitro* with Trx1, TrxR1, and NADPH as well as Grx1, GSH, GR, and NAPDH (Schweizer *et al.* 2014*a*). Under these conditions, nanomolar concentrations of protein thiols are sufficient to sustain catalysis. However, resulting activities appear more physiological and are lower than those obtained with DTT. In addition, millimolar concentrations of DTT may replace the endogenous thiols and thereby obfuscate their physiological roles by engaging the enzymes in non-physiological mechanisms (Croteau *et al.* 1998).

Interestingly, comparing the mDio3^{cat} structure to the structurally related Prx proteins revealed that both conserved deiodinase Cys are positioned in a way suggesting that they can act in a similar manner to the 'vicinal' and 'proximal' Cys residues in Prx proteins. Based on this close relationship to Prx, we have thus proposed a mechanism during which the selenenyliodide is attacked by an endogenous thiol, forming a selenenylsulfide and eliminating iodide (or the hydroxyl group of selenenic acid from hydrolysis). The selenenylsulfide is then reduced by the other endogenous thiol, regenerating the reduced selenolate, and the resulting disulfide is finally reduced by the exogenous thiol co-substrate (Fig. 5A). Such a cascade was found in other thiol peroxidases, and thioredoxin
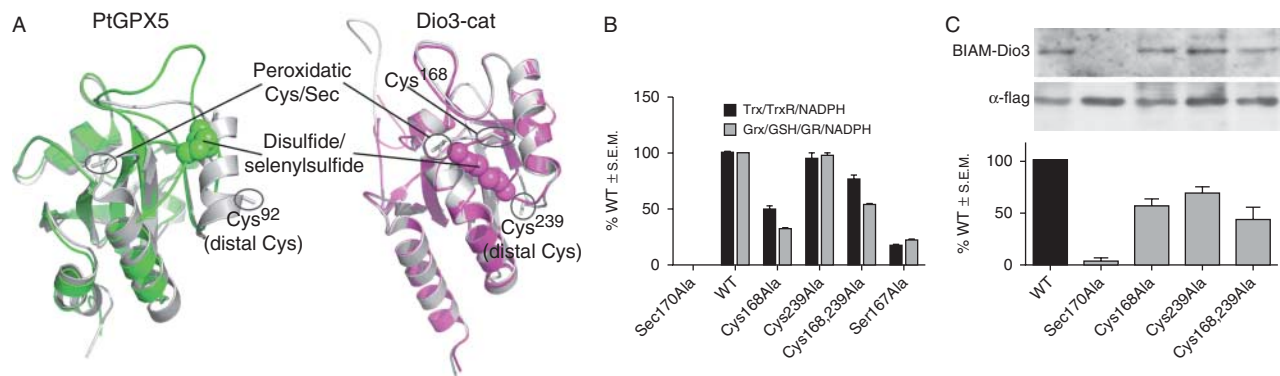

Figure 5

The role of conserved cysteines in deiodinase catalysis. (A) Comparison of reduced and oxidized states for PtGPX5 (left) and mDio3^{cat} (right). The structure of the oxidized form of PtGPX5 (green) was experimentally determined and requires only small rearrangements from the reduced state (grey) besides partial unwinding of the helix harboring Cys92. For mDio3^{cat}, an analogous oxidized state (magenta) could be modeled, starting with the experimental structure of the reduced form (grey), with even smaller rearrangements. (B) Impact of mutating conserved cysteines in Dio3 when protein thiols and regenerating systems are provided as reducing cofactors. Mutation of Cys^{239} does not reduce enzymatic activity. (C) Probing the reactivity of the selenol with biotinylated iodoacetamide (BIAM). Mutation of Cys^{239} does reduce the availability of the selenol suggesting a role for both cysteines in selenol reduction. Modified, with permission, from Schweizer U, Schlicker C, Braun D, Köhrle J & Steegborn C 2014*a* The crystal structure of mammalian selenocysteine-dependent iodothyronine deiodinase suggests a peroxiredoxin-like catalytic mechanism. *PNAS* **111** 10526–10531.

reductase even shows such a role of an oxidized active site Sec as starting point of the cascade (Zhong *et al.* 2000, Flohé *et al.* 2011). There is indeed experimental evidence for a contribution from both Cys, although replacing Cys[239] with Ala does not change enzyme activity, even in the presence of a Trx1 regenerating system (Fig. 5B; Schweizer *et al.* 2014a). The contribution of Cys[239] was apparent when the reactivity of a Sec[170] selenolate was probed using biotinylated iodoacetamide (Fig. 5C). In Dio3 oxidized through T_4 turnover, the reduced selenolate was again available. Together with mutagenesis effects for Cys[168] and/or Cys[239] on selenol labeling, this finding suggests that both Cys might react with the selenenyliodide and that they jointly regenerate the selenolate by formation of an endogenous disulfide. This internal deiodinase disulfide is then reduced by the thiol co-substrate (Schweizer *et al.* 2014a). Such an internal disulfide was also supported by effects of these mutations on cross-linking Dio3 with a Trx-Cys35Ser variant (Schweizer *et al.* 2014a). The lack of conservation of the two corresponding Cys in the *Amphioxus* Cys-deiodinase suggests that direct reduction of the catalytic residue is also an option (Klootwijk *et al.* 2011), but it might be sufficiently efficient only in such more distantly related, Cys- rather than Sec-based enzymes. In the proposed mechanism for mammalian deiodinases, uncertainty persists as to which thiol would act first and react with the selenenyliodide. Cys[168] is positioned closer to Sec[170] and deiodinase activity was always more affected when the vicinal Cys was removed (Croteau *et al.* 1998, Kuiper *et al.* 2002, Schweizer *et al.* 2014a), which might suggest preferential formation of a Cys[168]–Sec[170] selenenylsulfide. However, based on the structural homology to PtGPX5, for which an experimental structure of an oxidized state has been solved (Koh *et al.* 2007), we have modeled a Sec[170]–Cys[239] selenenylsulfide in mDio3[cat] (Schweizer *et al.* 2014a) (Fig. 5B). Dio3-Cys[239] is located close to the position of the PtGPX5 distal Cys, and the rearrangements required for disulfide/selenylsulfide formation with the catalytic Cys/Sec are rather small. The Dio3 structure, in fact, indicates flexibility in the involved regions, which would allow such a rearrangement (Schweizer *et al.* 2014a). Both Cys residues thus might be able to react with the selenenyliodide first. From a structural point of view, a Cys[168]–Cys[239] disulfide appears slightly more accessible for reduction than a Cys[239]–Sec[170] or Cys[168]–Sec[170] selenenylsulfide (Fig. 5C). The subsequent isomerization of either selenenylsulfide thus will facilitate the ultimate reaction with the thiol cofactor. Our interpretation of these findings is that the intermediate Sec[170]–Cys[239] or Cys[168]–Sec[170]

selenenylsulfide can be formed for protecting the oxidized enzyme from overoxidation at the expense of speed, since the following isomerization slows down the regeneration by the cofactor. This protective function for the selenenylsulfide is proposed in analogy to findings for Prx from *Trypanosoma* and *Aeropyrum pernix* (Jeon & Ishikawa 2003, Flohé *et al.* 2011). This model is in fact based on a large body of additional data for Prx proteins (Flohé *et al.* 2011) and explains why these Cys residues are conserved but mutating them shows effects only in some experimental setups. In particular for deiodinases, it would explain why mutating the conserved Cys does not significantly affect activity with the widely used, artificial thiol cofactor DTT (Croteau *et al.* 1998), since the high concentrations of this small reductant likely reduce the selenenyliodide directly.

In Dio2, which lacks the vicinal Cys, the selenenylsulfide would be long-lived and may constitute a molecular memory (Fig. 5B). Its conformation might target the enzyme for the observed ubiquitination that likely causes the substrate-induced degradation after completing one reaction cycle (Steinsapir *et al.* 2000; see below). Interestingly, the model of oxidized Dio2 featuring a sterically less accessible selenenylsulfide like the one in oxidized PtGPX5 also suggests an explanation why Dio2 is not reduced by the bulky GSH (Kuiper *et al.* 2002), but by the smaller DTT which is used for *in vitro* assays. Consistent with a role for the vicinal Cys missing in Dio2 in forming the efficiently reduced species, replacing the Ala residue found in Dio2 at this position with Cys improved is interaction with the reducing cofactor DTT (Kuiper *et al.* 2002). Why GSH was still not a co-factor for Dio2-Ala131Cys may be related to several small Dio2-specific insertions.

Clearly, there are still open questions regarding the role of endogenous thiols in deiodinase catalysis or regeneration, and the proposed Prx-like enzyme reduction mechanism remains to be further tested. Unfortunately, thiol/selenol chemistry cannot readily be followed by spectroscopic methods like absorbance and electron paramagnetic resonance (EPR), hampering progress in comparison to other dehalogenase mechanisms. Technically demanding approaches such as structural studies on intermediates and site-resolved mass spectrometry will be required for further analyzing the enzyme reduction half-cycle of deiodinase catalysis.

Autoinhibition of the monomer   Given the reactivity of selenolates and the structural similarity of deiodinases to peroxidases, it is rather surprising that mammalian deiodinases do not exhibit peroxidase activity. For example, a simple replacement of the

catalytic Ser by Sec in the serine protease subtilisin, which is structurally unrelated to known peroxidases, was sufficient to turn the enzyme into an efficient peroxidase (Wu & Hilvert 1990). Hydrogen peroxide is certainly more abundant than THs (and peroxidases), so that deiodinases need to be protected from performing competing reactions. In the mDio3[cat] structure, the selenolate from Sec[170] is shielded from the solvent by Pro[171], Pro[172], and Phe[258] (Fig. 6A). Phe[258] from the α2/β3-loop blocks the substrate binding groove in this monomer structure of apo mDio3[cat] (Schweizer *et al.* 2014a). The Phe[258] residue assumes a strained conformation indicating a switch function for this residue and the whole α2/β3-loop. This loop is known to participate in substrate binding in other Trx-fold proteins (Martin 1995). We speculate that dimerization and/or substrate binding releases the loop from the observed, protected, and autoinhibited conformation.

Dimerization The N-terminal region of deiodinases seems to contribute to proper cellular localization (Olvera *et al.* 2015), but it is also required for enzymatic activity, possibly through its contribution to formation of the physiological homodimer (Sagar *et al.* 2008). In contrast to the dimeric full-length deiodinase, mDio3[cat] behaved mainly monomeric with only a small fraction of dimeric protein (Schweizer *et al.* 2014a), indicating only a weak dimerization capability. This observation supports the previous conclusion that deiodinase dimerization is mainly mediated by the transmembrane region and apparently a contribution from the linker region (Sagar *et al.* 2008). The linker also influences the reaction specificity (Olvera *et al.* 2015), indicating that it contributes to the close coupling between dimerization and activity (see also below).

A deiodinase region around α3 and β4 comprises a cluster of residues conserved among deiodinase enzymes and exposed to the monomer surface. This region mediates dimerization in other redoxins, and a Dio3[cat] dimer was modeled based on this dimerization mode (Schweizer *et al.* 2014a). In this deiodinase dimer model, contacts between the N-terminal loop and β4 of one monomer and the β4/α3-loop and substrate site of the second monomer suggest how dimerization might relax the α2/β3-loop (Schweizer *et al.* 2014a). The mDio3[cat] structure shows that part of the conserved so-called 'deiodinase dimerization domain' (DDD), and in particular its IleTyrIle[196–198] motif (Leonard *et al.* 2005, Simpson *et al.* 2006; Fig. 4A), is embedded within the hydrophobic core of the catalytic domain monomer. While a major refolding of the Dio[cat] upon dimerization cannot be fully excluded, this arrangement suggests that this part of the DDD contributes to the stability of the individual catalytic domain and only indirectly to dimerization. The C-terminal part of the DDD, comprising a conserved GluAlaHis[202]XxxXxxAspGlyTrp motif (Fig. 4A; Leonard
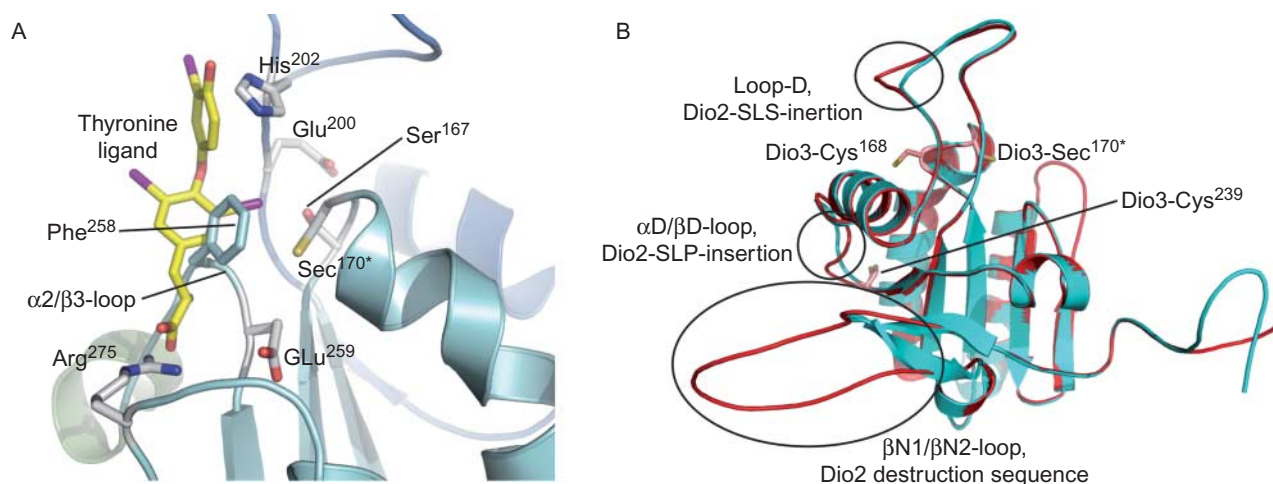

Figure 6
Models based on mDio3[cat]. (A) Model of a mDio3[cat] complex with a thyronine ligand. The figure shows that Phe[258] assumes an auto-inhibitory position preventing the access of substrates to the active site Sec[170]. (B) Model of mDio2 (red) based on mDio3[cat] (cyan). The Dio2-specific insertions are shown as modeled loops. Reproduced, with permission, from Schweizer U, Schlicker C, Braun D, Köhrle J & Steegborn C 2014a The crystal structure of mammalian selenocysteine-dependent iodothyronine deiodinase suggests a peroxiredoxin-like catalytic mechanism. *PNAS* **111** 10526–10531.

et al. 2005, Simpson *et al.* 2006), is part of the deiodinase-specific insertion (D-loop). It forms a potential lid above the active site and might participate in dimerization-induced rearrangements. Based on the modeled dimer, it would also not directly contribute to the dimer interface (Schweizer *et al.* 2014*a*), and its effect on dimerization would either be indirect or entail an interaction with the linker region. Proposing such a linker/lid model is tempting since it could explain the influence of the linker on the substrate specificity (Kuiper *et al.* 2003*b*, Olvera *et al.* 2015). However, the mode of deiodinase dimerization and why dimerization is a prerequisite for deiodinase activity remains speculative and awaits to be clarified through structural characterization of a dimeric full-length deiodinase enzyme.

Substrate binding and inner ring vs outer ring deiodination Conservation of structurally and catalytically critical residues show that basic architecture of the catalytic domains and key aspects of catalysis are conserved between deiodinase isoforms. A major difference between these isoforms is their substrate selectivity. In order to compare deiodinase sequences in relation to structure, we generated homology models for Dio1/2^{cat} based on the experimental Dio3^{cat} structure (Schweizer *et al.* 2014*a*; Fig. 6B).

While Dio1^{cat} and Dio3^{cat} are overall very similar, several structural features are specific for Dio2. Obvious are extensions in loop-D, at the α1 C-terminus, and in the βN1/βN2 loop (Fig. 6B). These insertions may contribute to interactions with ubiquitin ligase complexes (see above), underlining the need for an experimental structure of Dio2. Around the active sites, however, most residues even beyond the catalytically essential ones are conserved, including, e.g., a loop sequence conserved even in *Amphioxus* Cys-deiodinase (IleXxxGluAlaHisXxxSer-AspGlyTrp). Other features thus have to determine the regioselectivities of the isoforms.

The linker region between the membrane anchor and the catalytic domain has repeatedly been implicated in substrate interactions. Phe^{65} in human Dio1 is important for 5′-deiodination of rT$_3$ and 3,3′-T$_2$, but not T$_3$ and T$_4$ (Toyoda *et al.* 1994, 1997). It has been speculated that Phe^{65} interacts with the inner ring if a 5-iodine is lacking. Feline Dio1 differs from human Dio1 in this linker region and amino acid changes correlate with differential activity towards rT$_3$ (Kuiper *et al.* 2003*b*). Our crystal structure of mDio3^{cat} starts only a few amino acids C-terminal from the position corresponding to Dio1-Phe^{65} (Schweizer *et al.* 2014*a*). In mDio3^{cat}, this part is in an extended

conformation. It is tempting to speculate that in the full-length protein the linker folds back over the substrate-binding groove thus modulating substrate interactions.

How might the iodothyronine substrate be oriented in the active site? Because there is no enzyme/substrate structure available, we have modeled the iodothyronine ligand between His^{202} and Arg^{275} into the mDio3^{cat} structure (Schweizer *et al.* 2014*a*). This orientation is based on the T$_3$ binding mode in the TH receptors (Nascimento *et al.* 2006) where the His interacts with the thyronine 4′-OH and the Arg with the amino acid carboxylate. Interactions of His with the 4′-hydroxyl group and of Arg with the carboxylate are also observed in other iodothyronine-binding proteins (Schweizer *et al.* 2014*b*). Such a binding mode in deiodinases would bring the 5-iodine into close proximity with the Sec. Arg^{275} is replaced by a Lys in Dio2 within a Gly-Gly-Arg/Lys-Gly-Pro motif conserved in Dio3 and Dio2 and replaced by Gly-Lys-Ser/Ala/Pro-Gly-Pro in Dio1. These small isoenzyme differences could lead to conformational differences in the bound substrate. Recently, it was shown that the conformation of the thyronine amino acid moiety influences the reactivities of the iodine-binding carbons and the authors hypothesized that deiodinase isoenzymes may control regioselectivity via this mechanism (Mondal & Mugesh 2015). However, deiodinases can also act on substrates lacking either the carboxylate or the amino group, thyronamines and thyroacetic acids respectively (Piehl *et al.* 2008*a*,*b*, Klootwijk *et al.* 2011); therefore, the interaction with either part of the substrate amino acid moiety does not appear to be vital for catalysis.

For Dio2, ligand binding in the reverse orientation has been proposed, with the carboxylate close to His (Callebaut *et al.* 2003). Such an orientation would be consistent with covalent crosslinking experiments with Dio1 and *N*-BrAcetyl-T$_4$ (Köhrle *et al.* 1990). It is tempting to speculate that in principle both orientations may be possible depending on substrate and isoenzyme, and that the flexible linker may act as a lid covering the ligand in its binding site and dictates its orientation.

Substrate-induced inactivation Dio2 is well known for its activity-induced inactivation (Steinsapir *et al.* 1998). The inactivation process involves ubiquitination of the active enzyme, which leads to an inactive Dio2 conformation (Sagar *et al.* 2007), and is followed by proteasomal degradation (Steinsapir *et al.* 1998, 2000) or reactivation through deubiquitination (Sagar *et al.* 2007). Several proteins are involved in ubiquitinylation of Dio2 (Dentice *et al.* 2005) and a still unresolved question is how the

ubiquitin-ligase is able to distinguish a Dio2 from a Dio2 that has already seen the substrate. It is known that the first step for inactivation is actually turnover of a thyronine molecule (Steinsapir et al. 2000), and we suggest that the selenenylsulfide stabilizes a conformation that enhances recognition by the ubiquitin ligase. The inactivation loop and other insertions in Dio2 may also contribute to the interaction with the ubiquitin-ligase.

Similar observations were made with Dio1, which has been shown to exist in a substrate-induced inactive form (Zhu et al. 2012). Substrate-induced inactivation increased the fluorescence resonance energy transfer (FRET) signal in Dio1 fusion proteins indicating a conformational change in the inactivated dimer. Both inactivation and increased FRET signal depended on a functional active site (Zhu et al. 2012), suggesting that substrate turnover, rather than binding, is required for inactivation and is compatible with a selenenylsulfide contributing to this mechanism.

Deiodinase modulators and further drug development

As key enzymes in TH metabolism, all deiodinase isoforms might be suitable as drug targets. Deviations from normal TH plasma levels result in thyrotoxicosis or hypothyroidism, severe diseases which massively impair the patients' well-being. While hypothyroidism can be treated reasonably well by hormone replacement therapy, thyrotoxicosis may develop into life-threatening disease, and apart from pharmacological blockade of thyroidal TH biosynthesis and release with drugs that have known side effects, thyroidectomy exists as a last therapeutic resort (Mandel et al. 2011). Inhibition of Dio1 could provide an alternative therapeutic option for management of hyperthyroidism/thyrotoxicosis, if T_4 to T_3 conversion in thyroid or liver cells contributes to the pathomechanism (Koenig 2005). Increasing TH action through inhibition of Dio1/3-mediated T_3 inactivation may be a way to increase
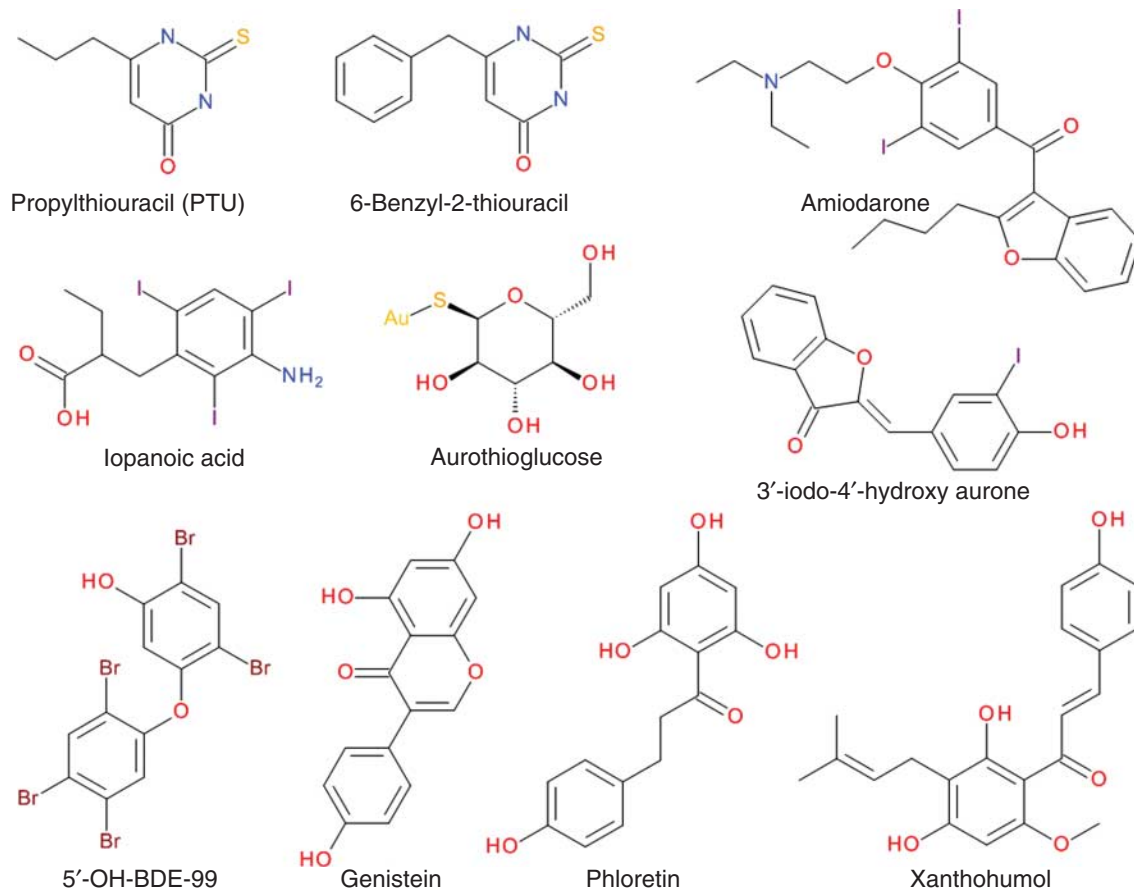

Figure 7
Compounds with deiodinase inhibiting activity.

Published by Bioscientifica Ltd.

energy expenditure in overweight patients, if the energy consuming effects of TH could be targeted to liver, muscle, and brown adipose tissue, while avoiding the heart. Furthermore, since Dio3 activity is required for strong proliferation, e.g. in developing tissue, and Dio3 is indeed up-regulated in some highly proliferative cancers, reducing Dio3 activity appears attractive for retarding cancer growth (Dentice *et al*. 2013*a*, Ciavardelli *et al*. 2014).

Pharmacological Dio inhibitors would be interesting leads for drug development, and they would also be valuable tools for *in vivo* studies. Few deiodinase-targeting compounds are available, however. The Dio1 inhibitor PTU (Fig. 7) is clinically used for treatment of hyperthyroidism but shows significant side effects (Glinoer & Cooper 2012). PTU and the closely related methylthiouracil (MTU) are selective for human Dio1 and are assumed to react with the oxidized active site selenenyl resulting from substrate deiodination (Kuiper *et al*. 2005*a*). Dio2/3 are PTU insensitive, likely due to an active site substitution that renders their selenyl residue less accessible (see above). However, PTU/MTU also inhibit thyroid peroxidase (TPO), which catalyzes iodination of thyroglobulin as a step in T_4 biosynthesis (Manna *et al*. 2013). The anti-thyroid effect of the related, clinically used compound methimazole, which inhibits TPO but shows no significant inhibition of Dio1, suggest that TPO inhibition is in fact the essential activity for the therapeutic effect. Nevertheless, PTU/MTU are valuable leads for the development of Dio inhibitors, and evaluating selenium analogs and a variety of modified thionamides indeed yielded the potent Dio1 inhibitor 6-benzyl-2-thiouracil (IC$_{50}$ 0.12 μM; Fig. 7; Rijntjes *et al*. 2013). This compound again showed no significant effect on Dio2, but the specificity for Dio1 vs TPO and other potential targets remains to be analyzed.

Dio2 is inhibited by the anti-arrhythmic drug amiodarone (Rosene *et al*. 2010), but its pleiotropic effects and targets preclude its use as anti-Dio2 drug. Two other inhibitors available for Dio2 and Dio3 are iopanoic acid and aurothioglucose (Kaplan & Utiger 1978, Berry *et al*. 1991; Fig. 7). Both compounds are non-specific, however, since they also act on Dio1 and possible other protein families. Aurothioglucose shows some differences in potency against the three Dio isoforms but appears not suitable for further development into a more specific compound since its inhibitory effect seems to be based on providing gold ions for the covalent modification of the Dio active site Sec in its reduced state (Kuiper *et al*. 2005*a*). Iopanoic acid resembles part of the thyronine substrate, should be a more specific ligand and thus might be more interesting as a starting point for further development, but

the compound itself shows little specificity and even serves as weak substrate at least for Dio1 (Renko *et al*. 2012). Similar features – limited specificity and possibly even enzymatic conversion – might apply to some of the other halogenated compounds that were described as Dio inhibitors. They belong to a variety of Dio inhibitory compounds that resulted from mainly exploratory studies on substances such as thyronine analogs, plant metabolites, halogenated dyes and phenolic xenobiotics (see e.g. Auf'mkolk *et al*. (1986*a*), Ferreira *et al*. (2002) and Shimizu *et al*. (2013)). An example for a potent Dio inhibitor from these studies is the 5'-OH derivative of the flame retardant BDE-99 (Fig. 7; Butt *et al*. 2011). As in most of these studies, inhibition of cellular lysates rather than isolated Dio isoforms was studied, and the structure–activity relationship was restricted to a smaller number of compounds, since they aimed at the identification of effects of nutrients and xenobiotics on TH metabolism and function. These limitations and the lack of detailed information on molecular interactions with the Dio enzyme have left most studies descriptive, without follow-up efforts on developing compounds into potent and specific Dio inhibitors. Other examples of such compounds are the plant derived phloretin (K_i=0.75 μM; Auf'mkolk *et al*. 1986*b*), the flavonoid baicalein (IC$_{50}$ 11 μM; Ferreira *et al*. 2002), and the aurone derivative 3'-iodo-4'-hydroxy aurone (IC$_{50}$ 0.5 μM; Auf'mkolk *et al*. 1986*a*; Fig. 7). A more recent study examined compound effects on all three Dio isoforms individually and revealed xanthohumol as an inhibitor with micromolar potency on Dio1/2/3 (IC$_{50}$ 1.5–3 μM), and genistein as a compound with similar potency (IC$_{50}$ 3 μM), but specific for Dio1 (Renko *et al*. 2015; Fig. 7).

The recently solved structure of Dio3 catalytic domain (Schweizer *et al*. 2014*a*) might now accelerate the development of Dio modulators by allowing structure-assisted approaches and the reanalysis of the described compounds and inhibition data. Such molecular analyses should soon provide new and promising candidate compounds for Dio inhibition for therapy and for functional studies. Further structural and mechanistic information on Dio action, such as the details of Dio dimerization or its interaction with thiol cofactors, might reveal additional opportunities and binding sites for Dio modulation and promise further insights in a unique biological reaction.

Journal of Molecular Endocrinology

References

Auf'mkolk M, Koehrle J, Hesch RD & Cody V 1986*a* Inhibition of rat liver iodothyronine deiodinase. Interaction of aurones with the iodothyronine ligand-binding site. *Journal of Biological Chemistry* **261** 11623–11630.

Auf'mkolk M, Koehrle J, Hesch RD, Ingbar SH & Cody V 1986*b* Crystal structure of phlorizin and the iodothyronine deiodinase inhibitory activity of phloretin analogues. *Biochemical Pharmacology* **35** 2221–2227. (doi:10.1016/0006-2952(86)90595-2)

Bayse CA & Rafferty ER 2010 Is halogen bonding the basis for iodothyronine deiodinase activity? *Inorganic Chemistry* **49** 5365–5367. (doi:10.1021/ic100711n)

Berry MJ, Kieffer JD & Larsen PR 1991 Evidence that cysteine, not selenocysteine, is in the catalytic site of type II iodothyronine deiodinase. *Endocrinology* **129** 550–552. (doi:10.1210/endo-129-1-550)

Berry MJ, Maia AL, Kieffer JD, Harney JW & Larsen PR 1992 Substitution of cysteine for selenocysteine in type I iodothyronine deiodinase reduces the catalytic efficiency of the protein but enhances its translation. *Endocrinology* **131** 1848–1852. (doi:10.1210/endo.131.4.1396330)

Bhat GB, Iwase K, Hummel BC & Walfish PG 1989 Kinetic characteristics of a thioredoxin-activated rat hepatic and renal low-Km iodothyronine 5′-deiodinase. *Biochemical Journal* **258** 785–792. (doi:10.1042/bj2580785)

Bianco AC & Kim BW 2006 Deiodinases: implications of the local control of thyroid hormone action. *Journal of Clinical Investigation* **116** 2571–2579. (doi:10.1172/JCI29812)

Bommer M, Kunze C, Fesseler J, Schubert T, Diekert G & Dobbek H 2014 Structural basis for organohalide respiration. *Science* **346** 455–458. (doi:10.1126/science.1258118)

Butt CM, Wang D & Stapleton HM 2011 Halogenated phenolic contaminants inhibit the *in vitro* activity of the thyroid-regulating deiodinases in human liver. *Toxicological Sciences* **124** 339–347. (doi:10.1093/toxsci/kfr117)

Callebaut I, Curcio-Morelli C, Mornon JP, Gereben B, Buettner C, Huang S, Castro B, Fonseca TL, Harney JW, Larsen PR *et al.* 2003 The iodothyronine selenodeiodinases are thioredoxin-fold family proteins containing a glycoside hydrolase clan GH-A-like structure. *Journal of Biological Chemistry* **278** 36887–36896. (doi:10.1074/jbc.M305725200)

Carreras CW & Santi DV 1995 The catalytic mechanism and structure of thymidylate synthase. *Annual Review of Biochemistry* **64** 721–762. (doi:10.1146/annurev.bi.64.070195.003445)

Ciavardelli D, Bellomo M, Crescimanno C & Vella V 2014 Type 3 deiodinase: role in cancer growth, stemness, and metabolism. *Frontiers in Endocrinology* **5** 215. (doi:10.3389/fendo.2014.00215)

Crooks GP, Xu L, Barkley RM & Copley SD 2010 Exploration of possible mechanisms for 4-chlorobenzoyl CoA dehalogenase: evidence for an aryl-enzyme intermediate. *Journal of the American Chemical Society* **117** 10791–10798. (doi:10.1021/ja00149a001)

Croteau W, Bodwell JE, Richardson JM & St Germain DL 1998 Conserved cysteines in the type 1 deiodinase selenoprotein are not essential for catalytic activity. *Journal of Biological Chemistry* **273** 25230–25236. (doi:10.1074/jbc.273.39.25230)

Dentice M, Antonini D & Salvatore D 2013*a* Type 3 deiodinase and solid tumors: an intriguing pair. *Expert Opinion on Therapeutic Targets* **17** 1369–1379. (doi:10.1517/14728222.2013.833189)

Dentice M, Marsili A, Zavacki A, Larsen PR & Salvatore D 2013*b* The deiodinases and the control of intracellular thyroid hormone signaling during cellular differentiation. *Biochimica et Biophysica Acta* **1830** 3937–3945. (doi:10.1016/j.bbagen.2012.05.007)

Dentice M, Bandyopadhyay A, Gereben B, Callebaut I, Christoffolete MA, Kim BW, Nissim S, Mornon JP, Zavacki AM, Zeold A *et al.* 2005 The Hedgehog-inducible ubiquitin ligase subunit WSB-1 modulates thyroid hormone activation and PTHrP secretion in the developing growth plate. *Nature Cell Biology* **7** 698–705. (doi:10.1038/ncb1272)

Ferreira AC, Lisboa PC, Oliveira KJ, Lima LP, Barros IA & Carvalho DP 2002 Inhibition of thyroid type 1 deiodinase activity by flavonoids. *Food and Chemical Toxicology* **40** 913–917. (doi:10.1016/S0278-6915(02)00064-9)

Flohé L, Toppo S, Cozza G & Ursini F 2011 A comparison of thiol peroxidase mechanisms. *Antioxidants & Redox Signaling* **15** 763–780. (doi:10.1089/ars.2010.3397)

Friedrichs B, Tepel C, Reinheckel T, Deussing J, von Figura K, Herzog V, Peters C, Saftig P & Brix K 2003 Thyroid functions of mouse cathepsins B, K, and L. *Journal of Clinical Investigation* **111** 1733–1745. (doi:10.1172/JCI15990)

Glinoer D & Cooper DS 2012 The propylthiouracil dilemma. *Current Opinion in Endocrinology, Diabetes, and Obesity* **19** 402–407. (doi:10.1097/MED.0b013e3283565b49)

Gnidehou S, Caillou B, Talbot M, Ohayon R, Kaniewski J, Noel-Hudson MS, Morand S, Agnangji D, Sezan A, Courtin F *et al.* 2004 Iodotyrosine dehalogenase 1 (DEHAL1) is a transmembrane protein involved in the recycling of iodide close to the thyroglobulin iodination site. *FASEB Journal* **18** 1574–1576. (doi:10.1096/fj.04-2023fje)

Goemann IM, Gereben B, Harney JW, Zhu B, Maia AL & Larsen PR 2010 Substitution of serine for proline in the active center of type 2 iodothyronine deiodinase substantially alters its *in vitro* biochemical properties with dithiothreitol but not its function in intact cells. *Endocrinology* **151** 821–829. (doi:10.1210/en.2009-0980)

Goswami A & Rosenberg IN 1977 Studies on a soluble thyroid iodotyrosine deiodinase: activation by NADPH and electron carriers. *Endocrinology* **101** 331–341. (doi:10.1210/endo-101-2-331)

Goswami A & Rosenberg IN 1985 Purification and characterization of a cytosolic protein enhancing GSH-dependent microsomal iodo-thyronine 5′-monodeiodination. *Journal of Biological Chemistry* **260** 6012–6019.

Goto K, Sonoda D, Shimada K, Sase S & Kawashima T 2010 Modeling of the 5′-deiodination of thyroxine by iodothyronine deiodinase: chemical corroboration of a selenenyl iodide intermediate. *Angewandte Chemie* **49** 545–547. (doi:10.1002/anie.200905796)

Hartmann N 1950 Über den Abbau von Dijodtyrosin im Gewebe. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **285** 1–17.

Hu J, Chuenchor W & Rokita SE 2015 A switch between one- and two-electron chemistry of the human flavoprotein iodotyrosine deiodinase is controlled by substrate. *Journal of Biological Chemistry* **290** 590–600. (doi:10.1074/jbc.M114.605964)

Jeon SJ & Ishikawa K 2003 Characterization of novel hexadecameric thioredoxin peroxidase from *Aeropyrum pernix K1*. *Journal of Biological Chemistry* **278** 24174–24180. (doi:10.1074/jbc.M300618200)

Jonas W, Lietzow J, Wohlgemuth F, Hoefig CS, Wiedmer P, Schweizer U, Köhrle J & Schurmann A 2015 3,5-Diiodo-L-thyronine (3,5-T$_2$) exerts thyromimetic effects on hypothalamus–pituitary–thyroid axis, body composition, and energy metabolism in male diet-induced obese mice. *Endocrinology* **1** 389–399. (doi:10.1210/en.2014-1604)

Kaplan MM & Utiger RD 1978 Iodothyronine metabolism in rat liver homogenates. *Journal of Clinical Investigation* **61** 459–471. (doi:10.1172/JCI108957)

Klootwijk W, Friesema EC & Visser TJ 2011 A nonselenoprotein from *Amphioxus* deiodinates triac but not T$_3$: is triac the primordial bioactive thyroid hormone? *Endocrinology* **152** 3259–3267. (doi:10.1210/en.2010-1408)

Koenig RJ 2005 Regulation of type 1 iodothyronine deiodinase in health and disease. *Thyroid* **15** 835–840. (doi:10.1089/thy.2005.15.835)

Koh CS, Didierjean C, Navrot N, Panjikar S, Mulliert G, Rouhier N, Jacquot JP, Aubry A, Shawkataly O & Corbier C 2007 Crystal structures of a poplar thioredoxin peroxidase that exhibits the structure of glutathione peroxidases: insights into redox-driven conformational

changes. *Journal of Molecular Biology* **370** 512–529. (doi:10.1016/j.jmb.2007.04.031)

Köhrle J, Rasmussen UB, Ekenbarger DM, Alex S, Rokos H, Hesch RD & Leonard JL 1990 Affinity labeling of rat liver and kidney type I 5′-deiodinase. Identification of the 27-kDa substrate binding subunit. *Journal of Biological Chemistry* **265** 6155–6163.

Kuiper GG, Klootwijk W & Visser TJ 2002 Substitution of cysteine for a conserved alanine residue in the catalytic center of type II iodothyronine deiodinase alters interaction with reducing cofactor. *Endocrinology* **143** 1190–1198. (doi:10.1210/endo.143.4.8738)

Kuiper GG, Klootwijk W & Visser TJ 2003a Substitution of cysteine for selenocysteine in the catalytic center of type III iodothyronine deiodinase reduces catalytic efficiency and alters substrate preference. *Endocrinology* **144** 2505–2513. (doi:10.1210/en.2003-0084)

Kuiper GG, Wassen F, Klootwijk W, Van Toor H, Kaptein E & Visser TJ 2003b Molecular basis for the substrate selectivity of cat type I iodothyronine deiodinase. *Endocrinology* **144** 5411–5421. (doi:10.1210/en.2003-0728)

Kuiper GG, Kester MH, Peeters RP & Visser TJ 2005a Biochemical mechanisms of thyroid hormone deiodination. *Thyroid* **15** 787–798. (doi:10.1089/thy.2005.15.787)

Kuiper GG, Klootwijk W & Visser TJ 2005b Expression of recombinant membrane-bound type I iodothyronine deiodinase in yeast. *Journal of Molecular Endocrinology* **34** 865–878. (doi:10.1677/jme.1.01770)

Kuiper GG, Klootwijk W, Morvan Dubois G, Destree O, Darras VM, Van der Geyten S, Demeneix B & Visser TJ 2006 Characterization of recombinant *Xenopus laevis* type I iodothyronine deiodinase: substitution of a proline residue in the catalytic center by serine (Pro132Ser) restores sensitivity to 6-propyl-2-thiouracil. *Endocrinology* **147** 3519–3529. (doi:10.1210/en.2005-0711)

Leonard JL, Simpson G & Leonard DM 2005 Characterization of the protein dimerization domain responsible for assembly of functional selenodeiodinases. *Journal of Biological Chemistry* **280** 11093–11100. (doi:10.1074/jbc.M500011200)

Maia AL, Kim BW, Huang SA, Harney JW & Larsen PR 2005 Type 2 iodothyronine deiodinase is the major source of plasma T3 in euthyroid humans. *Journal of Clinical Investigation* **115** 2524–2533. (doi:10.1172/JCI25083)

Mandel SJ, Larsen PR & Davies TF 2011 Thyrotoxicosis. In *Williams Textbook of Endocrinology*. Eds S Melmed, KS Polonsky, PR Larsen & HM Kronenberg. Philadelphia, PA, USA: Elsevier Saunders.

Manna D & Mugesh G 2010 A chemical model for the inner-ring deiodination of thyroxine by iodothyronine deiodinase. *Angewandte Chemie* **49** 9246–9249. (doi:10.1002/anie.201005235)

Manna D & Mugesh G 2012 Regioselective deiodination of thyroxine by iodothyronine deiodinase mimics: an unusual mechanistic pathway involving cooperative chalcogen and halogen bonding. *Journal of the American Chemical Society* **134** 4269–4279. (doi:10.1021/ja210478k)

Manna D, Roy G & Mugesh G 2013 Antithyroid drugs and their analogues: synthesis, structure, and mechanism of action. *Accounts of Chemical Research* **46** 2706–2715. (doi:10.1021/ar4001229)

Manna D, Mondal S & Mugesh G 2015 Halogen bonding controls the regioselectivity of the deiodination of thyroid hormones and their sulfate analogues. *Chemistry* **21** 2409–2416. (doi:10.1002/chem.201405442)

Martin JL 1995 Thioredoxin – a fold for all reasons. *Structure* **3** 245–250. (doi:10.1016/S0969-2126(01)00154-X)

McTamney PM & Rokita SE 2009 A mammalian reductive deiodinase has broad power to dehalogenate chlorinated and brominated substrates. *Journal of the American Chemical Society* **131** 14212–14213. (doi:10.1021/ja906642n)

Mendoza A, Navarrete-Ramirez P, Hernandez-Puga G, Villalobos P, Holzer G, Renaud JP, Laudet V & Orozco A 2013 3,5-T2 is an alternative ligand for the thyroid hormone receptor β1. *Endocrinology* **154** 2948–2958. (doi:10.1210/en.2013-1030)

Mondal S & Mugesh G 2015 Structure elucidation and characterization of different thyroxine polymorphs. *Angewandte Chemie* **54** 10833–10837. (doi:10.1002/anie.201505281)

Moreno JC 2003 Identification of novel genes involved in congenital hypothyroidism using serial analysis of gene expression. *Hormone Research* **60** (Suppl 3) 96–102. (doi:10.1159/000074509)

Moreno JC & Visser TJ 2010 Genetics and phenomics of hypothyroidism and goiter due to iodotyrosine deiodinase (DEHAL1) gene mutations. *Molecular and Cellular Endocrinology* **322** 91–98. (doi:10.1016/j.mce.2010.03.010)

Moreno JC, Klootwijk W, van Toor H, Pinto G, D'Alessandro M, Leger A, Goudie D, Polak M, Gruters A & Visser TJ 2008 Mutations in the iodotyrosine deiodinase gene and hypothyroidism. *New England Journal of Medicine* **358** 1811–1818. (doi:10.1056/NEJMoa0706819)

Mullur R, Liu YY & Brent GA 2014 Thyroid hormone regulation of metabolism. *Physiological Reviews* **94** 355–382. (doi:10.1152/physrev.00030.2013)

Nascimento AS, Dias SM, Nunes FM, Aparicio R, Ambrosio AL, Bleicher L, Figueira AC, Santos MA, de Oliveira NM, Fischer H et al. 2006 Structural rearrangements in the thyroid hormone receptor hinge domain and their putative role in the receptor function. *Journal of Molecular Biology* **360** 586–598. (doi:10.1016/j.jmb.2006.05.008)

Olvera A, Mendoza A, Villalobos P, Mayorga-Martinez L, Orozco A & Valverde RC 2015 The variable region of iodothyronine deiodinases directs their catalytic properties and subcellular localization. *Molecular and Cellular Endocrinology* **402** 107–112. (doi:10.1016/j.mce.2015.01.011)

Orozco A, Navarrete-Ramirez P, Olvera A & Garcia GC 2014 3,5-Diiodothyronine (T2) is on a role. A new hormone in search of recognition. *General and Comparative Endocrinology* **203** 174–180. (doi:10.1016/j.ygcen.2014.02.014)

Payne KA, Quezada CP, Fisher K, Dunstan MS, Collins FA, Sjuts H, Levy C, Hay S, Rigby SE & Leys D 2015 Reductive dehalogenase structure suggests a mechanism for B12-dependent dehalogenation. *Nature* **517** 513–516. (doi:10.1038/nature13901)

Piehl S, Heberer T, Balizs G, Scanlan TS & Köhrle J 2008a Development of a validated liquid chromatography/tandem mass spectrometry method for the distinction of thyronine and thyronamine constitutional isomers and for the identification of new deiodinase substrates. *Rapid Communications in Mass Spectrometry* **22** 3286–3296. (doi:10.1002/rcm.3732)

Piehl S, Heberer T, Balizs G, Scanlan TS, Smits R, Koksch B & Köhrle J 2008b Thyronamines are isozyme specific substrates of deiodinases. *Endocrinology* **149** 3037–3045. (doi:10.1210/en.2007-1678)

Renko K, Hoefig CS, Hiller F, Schomburg L & Köhrle J 2012 Identification of iopanoic acid as substrate of type 1 deiodinase by a novel nonradioactive iodide-release assay. *Endocrinology* **153** 2506–2513. (doi:10.1210/en.2011-1863)

Renko K, Schache S, Hoefig CS, Welsink T, Schwiebert C, Braun D, Becker NP, Köhrle J & Schomburg L 2015 An improved nonradioactive screening method identifies genistein and xanthohumol as potent inhibitors of iodothyronine deiodinases. *Thyroid* **25** 962–968. (doi:10.1089/thy.2015.0058)

Rijntjes E, Scholz PM, Mugesh G & Köhrle J 2013 Se- and s-based thiouracil and methimazole analogues exert different inhibitory mechanisms on type 1 and type 2 deiodinases. *European Thyroid Journal* **2** 252–258. (doi:10.1159/000355288)

Rokita SE, Adler JM, McTamney PM & Watson JA Jr 2010 Efficient use and recycling of the micronutrient iodide in mammals. *Biochimie* **92** 1227–1235. (doi:10.1016/j.biochi.2010.02.013)

Rosene ML, Wittmann G, Arrojo e Drigo R, Singru PS, Lechan RM & Bianco AC 2010 Inhibition of the type 2 iodothyronine deiodinase underlies the elevated plasma TSH associated with amiodarone treatment. *Endocrinology* **151** 5961–5970. (doi:10.1210/en.2010-0553)

Sagar GD, Gereben B, Callebaut I, Mornon JP, Zeold A, da Silva WS, Luongo C, Dentice M, Tente SM, Freitas BC et al. 2007 Ubiquitination-

induced conformational change within the deiodinase dimer is a switch regulating enzyme activity. *Molecular and Cellular Biology* **27** 4774–4783. (doi:10.1128/MCB.00283-07)

Sagar GD, Gereben B, Callebaut I, Mornon JP, Zeold A, Curcio-Morelli C, Harney JW, Luongo C, Mulcahey MA, Larsen PR *et al.* 2008 The thyroid hormone-inactivating deiodinase functions as a homodimer. *Molecular Endocrinology* **22** 1382–1393. (doi:10.1210/me.2007-0490)

Salvatore D, Davies TF, Schlumberger M-J, Hay ID & Larsen PR 2011 Thyroid physiology and diagnostic evaluation of patients with thyroid disorders. In *Williams Textbook of Endocrinology*, 12th edn. Eds S Melmed, KS Polonsky, PR Larsen & HM Kronenberg. Philadelphia, PA, USA: Elsevier Saunders.

Schweizer U, Weitzel JM & Schomburg L 2008 Think globally: act locally. New insights into the local regulation of thyroid hormone availability challenge long accepted dogmas. *Molecular and Cellular Endocrinology* **289** 1–9. (doi:10.1016/j.mce.2008.04.007)

Schweizer U, Schlicker C, Braun D, Köhrle J & Steegborn C 2014a The crystal structure of mammalian selenocysteine-dependent iodothyronine deiodinase suggests a peroxiredoxin-like catalytic mechanism. *PNAS* **111** 10526–10531. (doi:10.1073/pnas.1323873111)

Schweizer U, Johannes J, Bayer D & Braun D 2014b Structure and function of thyroid hormone plasma membrane transporters. *European Thyroid Journal* **3** 143–153. (doi:10.1159/000367858)

Shimizu R, Yamaguchi M, Uramaru N, Kuroki H, Ohta S, Kitamura S & Sugihara K 2013 Structure–activity relationships of 44 halogenated compounds for iodotyrosine deiodinase-inhibitory activity. *Toxicology* **314** 22–29. (doi:10.1016/j.tox.2013.08.017)

Simpson GI, Leonard DM & Leonard JL 2006 Identification of the key residues responsible for the assembly of selenodeiodinases. *Journal of Biological Chemistry* **281** 14615–14621. (doi:10.1074/jbc.M600783200)

Steinsapir J, Harney J & Larsen PR 1998 Type 2 iodothyronine deiodinase in rat pituitary tumor cells is inactivated in proteasomes. *Journal of Clinical Investigation* **102** 1895–1899. (doi:10.1172/JCI4672)

Steinsapir J, Bianco AC, Buettner C, Harney J & Larsen PR 2000 Substrate-induced down-regulation of human type 2 deiodinase (hD2) is mediated through proteasomal degradation and requires interaction with the enzyme's active center. *Endocrinology* **141** 1127–1135. (doi:10.1210/endo.141.3.7355)

Sun BC, Harney JW, Berry MJ & Larsen PR 1997 The role of the active site cysteine in catalysis by type 1 iodothyronine deiodinase. *Endocrinology* **138** 5452–5458. (doi:10.1210/endo.138.12.5623)

Tata JR 1968 Hormonal regulation of growth and protein synthesis. *Nature* **219** 331–337. (doi:10.1038/219331a0)

Thomas SR, McTamney PM, Adler JM, Laronde-Leblanc N & Rokita SE 2009 Crystal structure of iodotyrosine deiodinase, a novel flavoprotein responsible for iodide salvage in thyroid glands. *Journal of Biological Chemistry* **284** 19659–19667. (doi:10.1074/jbc.M109.013458)

Toyoda N, Harney JW, Berry MJ & Larsen PR 1994 Identification of critical amino acids for 3,5,3′-triiodothyronine deiodination by human type 1 deiodinase based on comparative functional–structural analyses of the human, dog, and rat enzymes. *Journal of Biological Chemistry* **269** 20329–20334.

Toyoda N, Kaptein E, Berry MJ, Harney JW, Larsen PR & Visser TJ 1997 Structure–activity relationships for thyroid hormone deiodination by mammalian type I iodothyronine deiodinases. *Endocrinology* **138** 213–219. (doi:10.1210/endo.138.1.4900)

Wu ZP & Hilvert D 1990 Selenosubtilisin as a glutathione peroxidase mimic. *Journal of the American Chemical Society* **112** 5647–5648. (doi:10.1021/ja00170a043)

Zhong L, Arner ES & Holmgren A 2000 Structure and mechanism of mammalian thioredoxin reductase: the active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine–selenocysteine sequence. *PNAS* **97** 5854–5859. (doi:10.1073/pnas.100114897)

Zhu B, Shrivastava A, Luongo C, Chen T, Harney JW, Marsili A, Tran TV, Bhadouria A, Mopala R, Steen AI *et al.* 2012 Catalysis leads to posttranslational inactivation of the type 1 deiodinase and alters its conformation. *Journal of Endocrinology* **214** 87–94. (doi:10.1530/JOE-11-0459)

Journal of Molecular Endocrinology