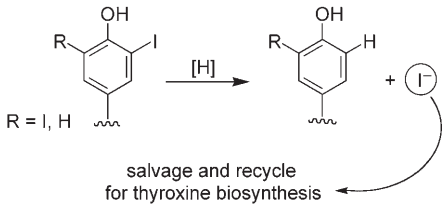
DOI: 10.1002/cbic.200700562

Flavoprotein Iodotyrosine Deiodinase Functions without Cysteine Residues

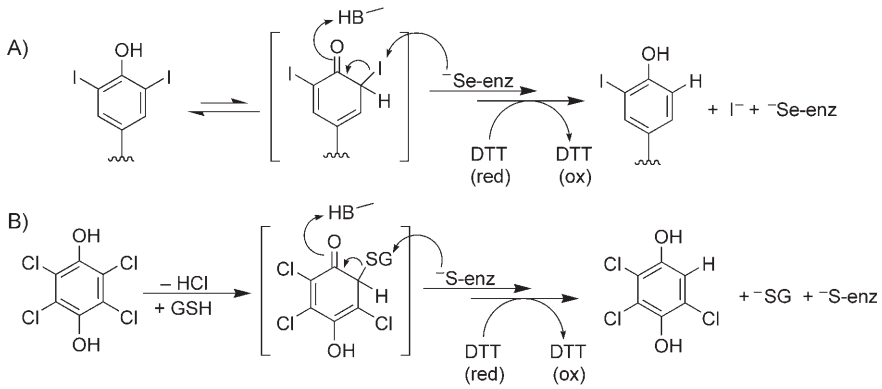
James A. Watson, Jr., Patrick M. McTamney, Jennifer M. Adler, and Steven E. Rokita*[a]

Flavoproteins are able to catalyze a variety of metabolic processes due to the wide range of reactions promoted by flavin.[1–4] Appreciation for the catalytic power of flavin continues to increase as still more flavin-dependent processes are discovered. Typically, once a new activity is detected in one organism, related reactions are often identified soon thereafter in numerous other organisms, as recently illustrated by the growing class of flavin-dependent halogenases.[5] In contrast, only one highly conserved flavoprotein has yet been directly associated with reductive dehalogenation. This enzyme, iodotyrosine deiodinase (IYD), is critical in the thyroid for recycling iodide from the by-products of thyroxine (T4, 4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenylalanine) biosynthesis (Scheme 1).



Scheme 1. Iodide homeostasis is maintained in part by recycling iodide from mono- and diiodotyrosine.

Reductive dehalogenation is unusual in aerobic life and particularly rare in higher organisms. Humans express only two representatives, the flavoprotein mentioned above that acts on iodinated tyrosine and a selenoprotein iodothyronine deiodinase (ID) that acts on T4 and its derivatives.[6] Similar mechanisms have been proposed for these enzymes based on their reported dependence on a cysteine and selenocysteine residue, respectively.[7–10] ID is thought to stabilize a nonaromatic tautomer of T4 and then release an equivalent of I^+ that oxidizes its selenocysteine residue (Scheme 2).[6] An analogous process of stabilizing a substrate tautomer and reacting with an active site cysteine has also been proposed for a reductive dechlorination catalyzed by the bacterial enzyme tetrachlorohydroquinone dehalogenase (TD) (Scheme 2).[11,12] Similarly, IYD appears to stabilize a nonaromatic tautomer of diiodotyrosine because a pyridone derivative that mimics the intermediate tautomer binds to the enzyme with very high affinity.[10] Subsequent evidence was then expected to implicate involvement of one or more cysteine residues in IYD turnover as well. The results described herein reveal instead that all aspects of turnover are independent of cysteine, and neither ID nor TD provides precedence for IYD.



Scheme 2. Proposed mechanisms of A) thyroxine deiodination promoted by iodothyronine deiodinase and B) tetrachlorohydroquinone dechlorination promoted by tetrachlorohydroquinone dehalogenase.

The major structural domain of IYD belongs to the NADH oxidase/flavin reductase superfamily, and prior modeling suggested that two of the three cysteines (C217 and C239, Figure 1) were located in the proposed active site adjacent to the bound flavin mononucleotide (FMN).[13] This proximity is reminiscent of the many flavoproteins that contain redox-active cysteines, and hence the flavin of IYD was originally expected only to mediate electron transfer to an active site cysteine. Although NADPH is thought to act as the physiological source of reducing equivalents, its effect is lost after detergent is used to disrupt the membrane surrounding IYD. Under these conditions, dithionite is required as an alternative electron donor.[7] Thiols such as GSH and DTT do not support turnover by IYD in contrast to their ability to promote deiodination by ID and dechlorination by TD.[6,11,12] Still, thiols are chemically

Figure 1. The amino acid sequence of iodotyrosine deiodinase contains three cysteines (C13, C217, and C239) and establishes three domains, a transmembrane domain (1–24) and inter-domain (25–82) and a flavin reductase/NADPH oxidase domain (83–285).

[a] *Dr. J. A. Watson, Jr., P. M. McTamney, J. M. Adler, Prof. S. E. Rokita*
Department of Chemistry and Biochemistry, University of Maryland
College Park, MD 20742 (USA)
Fax: (+1) 301-405-9376
E-mail: Rokita@umd.edu

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

competent to promote deiodination of iodotyrosine as demonstrated from previous model studies with cysteine.[14]

IYD has been notoriously difficult to purify from thyroids,[13,15] and its recent heterologous expression in mammalian cells (HEK293 and CHO)[13,16] provided the first opportunity to examine the function of each cysteine residue definitively. Attention was initially directed at the two possible active site cysteines. Two single mutants (C217A and C239A) and a double mutant (C217A/C239A) were generated in the full-length membrane-bound protein encoded by the *Mus musculus* gene. Contrary to all expectations based on the proposed mechanisms of ID and TD (Scheme 2), IYD maintained its deiodinase activity in the presence of dithionite despite removal of either one or both cysteines (Table 1). Previous modeling had suggested that C217 was the more distal of the cysteines with respect to the flavin,[13] and the kinetic constants for its mutant were nearly indistinguishable from wild-type. Mutation of the proximal C239 enhanced both K_M and k_{cat}, but the overall k_{cat}/K_M decreased by ~2.5-fold relative to wild-type. The double mutant exhibited properties intermediate between the two single mutants. These results refute any possibility that the cysteines are directly involved in substrate dehalogenation, and instead implicate a more active role for the flavin.

Table 1. Kinetic parameters of wild-type and mutant iodotyrosine deiodinases.[a]

IYD	Dithionite[b] K_M DIT [μM]	Dithionite[b] k_{cat} [min^{-1}]	Dithionite[b] k_{cat}/K_M [$min^{-1}\,\mu M^{-1}$]	NADPH[c] [units $nmol^{-1}$]
wild-type	8 ± 3[d]	7.1 ± 0.9	0.89	6.8 ± 0.4
C217A	13 ± 4	8.3 ± 0.6	0.64	9.0 ± 2
C239A	79 ± 7	28 ± 2	0.35	8.7 ± 0.6
C217A/C239A	42 ± 7	16 ± 2	0.38	7.7 ± 0.9
Δ2–33	6 ± 2	5.8 ± 0.6	0.95	1.3 ± 0.1

[a] Iodotyrosine deiodinase was transiently expressed in HEK293 cells. Cells were lysed and directly assayed for $^{125}I^-$ release from [^{125}I]diiodotyrosine under standard conditions (see Supporting Information for more details).[13,17] Lysates were also analyzed by denaturing polyacrylamide gel electrophoresis, Coomassie Brilliant Blue staining, and densitometry (Supporting Information). [b] Dithionite concentration was held constant at 1% (*w/v*), and diiodotyrosine (DIT) concentration was varied between 1.0 and 50 μM. [c] NADPH concentration was held constant at 10 mM. One unit $nmol^{-1}$ is defined as nanomoles of I^- released per h per nmol of IYD. Uncertainties derive from the standard deviation associated with fitting three or more independent data sets. [d] The large uncertainties resulted in part from substrate inhibition as reported earlier.[15]

The cysteine pair (C217, C239) is present in all homologous proteins so far identified in organisms that use thyroxine (zebra fish to mammals).[18] Conversely, these cysteines are not maintained in homologous proteins found in all other organisms including *Drosophila melanogaster* and *Caenorhabditis elegans*. Such a trend would almost certainly affirm some relevance to the cysteines, perhaps one that would not have been apparent from the dithionite-based assay. The physiological source of electrons for IYD likely derives from NADPH as mediated by a membrane-bound reductase. This process was initially circumvented by using dithionite as the reductant to focus on substrate dehalogenation rather than enzyme reduction. To expand the search for the role of cysteine in IYD, the wild-type and mutant enzymes were compared once more, but this time in the presence of NADPH rather than dithionite. Regardless of the cysteines, the single and double mutants exhibited activity equivalent to that of the wild-type enzyme under common conditions (Table 1). Thus, C217 and C239 have no clear function in IYD catalysis.

The physiological reductase required for transferring electrons from NADPH to IYD has yet to be identified, and its activity has only been observed with membrane-bound IYD.[7] Previous methods of detergent extraction were unable to distinguish between the possibilities of dissociating a discrete reductase from IYD or inactivating a reductase domain within IYD.[15] Recent results now suggest that the reductase is a separate polypeptide. The same reductase or an equivalent is coincidently present in HEK293 cells, as deiodination can be driven by NADPH in these cells after heterologous expression of IYD. In contrast, NADPH-dependent deiodination is not detected when IYD is expressed in CHO cells.[16] This lack of activity can now be ascribed to the absence of an effective reductase rather than a deficiency in IYD. Lysates of CHO cells expressing IYD promote deiodination of diiodotyrosine (DIT) in the presence of dithionite (1% *w/v*) with kinetic constants (K_M 7.1 ± 1 μM, k_{cat} 4.2 ± 0.2 min^{-1}, k_{cat}/K_M 0.59 $min^{-1}\,\mu M^{-1}$, Supporting Information) that are similar to those measured for IYD expressed in HEK 293 cells (Table 1). In contrast, these same lysates were confirmed to exhibit no ability to promote deiodination of DIT in the presence of NADPH (10 mM) at the limit of sensitivity (~5% of the rates measured in the presence of dithionite, Supporting Information). This lower limit of NADPH-dependent activity is also significantly lower than the weak but measurable NADPH activity of soluble IYD Δ2–33 and cell lysates of HEK containing the proposed membrane-bound reductase as described below.

An N-terminal truncation of IYD Δ2–33 was also constructed from the *M. musculus* gene to examine if NADPH-dependent activity was truly regulated through membrane localization. This domain, containing the remaining cysteine C13 (Figure 1), had been tentatively assigned as a membrane anchor based on its lipophilicity.[13] Removal of this domain successfully converted IYD from a membrane-associated form to a new soluble form. Expression of IYD Δ2–33 in HEK293 generated a protein that remained in the supernatant (S) after separating the microsomal fraction (P) by ultracentrifugation (lanes 5 and 6, Figure 2). In contrast, full-length IYD associated with microsomes.[13] The absence of its membrane anchor and membrane association did not substantially alter the kinetics of deiodination promoted by IYD Δ2–33 in the presence of dithionite. Neither K_M nor k_{cat} decreased by more than 25% relative to that exhibited by the membrane-bound wild-type IYD (Table 1). However, loss of the membrane anchor strongly suppressed the ability of IYD to use NADPH for deiodination in lysates of HEK293 cells by fivefold (Table 1). This confirms the importance of membrane association for effective interaction between IYD and the NADPH-dependent reductase and concurrently dem-

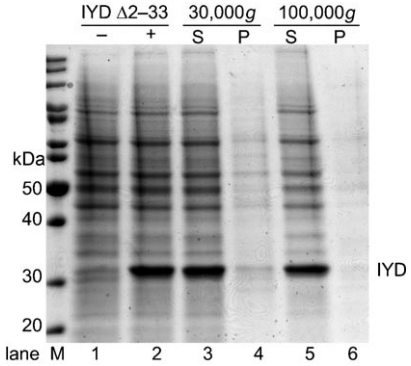


Figure 2. Removal of the N-terminal membrane anchor releases IYD from cell membranes. HEK293 cells lacking (lane 1) and containing the plasmid encoding IYD Δ2–33 (lane 2) were lysed and analyzed directly by denaturing gel electrophoresis and stained with Coomassie Brilliant Blue. Crude lysate containing IYD Δ2–33 was also centrifuged at 30000 *g* to yield a soluble fraction (S, lane 3) and a pellet of cell debris (P, lane 4). The soluble fraction was further centrifuged at 100000 *g* to separate soluble protein (S, lane 5) from membrane-bound protein (P, lane 6). In each case, pellets were resuspended in volumes equal to their corresponding supernatants. Molecular weight markers are included in lane M.

onstrates that C13, like C217 and C239, is superfluous to catalytic deiodination of diiodotyrosine.

Neither flavin reduction nor substrate dehalogenation require any of the three cysteines of IYD despite their absolute conservation throughout the relevant biological families. Accordingly, the chemical and biochemical precedence for promoting reductive dehalogenation by cysteine and related selenocysteine derivatives is irrelevant to IYD. A recent report indicates that certain amines may also promote reductive dehalogenation in a model system related to those containing cysteine,[19] and therefore the role of histidine and lysine residues in IYD also deserves scrutiny. Interestingly, an expected intermediate of such a process, lysine-εNH-Cl, has just been proposed to form during catalysis of the reverse process, a flavin-dependent halogenation.[20] Radical mechanisms analogous to that suggested for the flavoprotein chorismate synthase also deserve future attention.[21] Regardless of the mechanism, the repertoire of flavin chemistry should now be expanded to include reductive dehalogenation in the absence of redox-active thiols.

Acknowledgements

We greatly appreciate advice and help from Professor Iqbal Hamza and his research group (M. Winn and C. Hall, in particular).

Keywords: catalytic mechanisms · deiodination · flavoproteins · reductive dehalogenation · thiols

[1] V. Massey, *Biochem. Soc. Trans.* **2000**, *28*, 283–296.
[2] L. De Colibus, A. Mattevi, *Curr. Opin. Struct. Biol.* **2006**, *16*, 722–728.
[3] V. Joosten, W. J. H. van Berkel, *Curr. Opin. Chem. Biol.* **2007**, *11*, 195–202.
[4] S. O. Mansoorabadi, C. J. Thibodeaux, H.-W. Liu, *J. Org. Chem.* **2007**, *72*, 6329–6342.
[5] K.-H. van Pée, E. P. Patallo, *Appl. Microbiol. Biotechnol.* **2006**, *70*, 631–641.
[6] A. C. Bianco, D. Salvatore, B. Gereben, M. J. Berry, P. R. Larsen, *Endocr. Rev.* **2002**, *23*, 38–89.
[7] A. Goswami, I. N. Rosenberg, *Endocrinology* **1977**, *101*, 331–341.
[8] A. Goswami, I. N. Rosenberg, *J. Biol. Chem.* **1979**, *254*, 12326–12330.
[9] M. J. Berry, J. D. Kieffer, J. W. Harney, P. R. Larsen, *J. Biol. Chem.* **1991**, *266*, 14155–14158.
[10] M. Kunishima, J. E. Friedman, S. E. Rokita, *J. Am. Chem. Soc.* **1999**, *121*, 4722–4723.
[11] P. M. Kiefer, D. L. McCarthy, S. D. Copley, *Biochemistry* **2002**, *41*, 1308–1314.
[12] P. M. Kiefer, S. D. Copley, *Biochemistry* **2002**, *41*, 1315–1322.
[13] J. E. Friedman, J. A. Watson, Jr., D. W.-H. Lam, S. E. Rokita, *J. Biol. Chem.* **2006**, *281*, 2812–2819.
[14] K. Hartmann, N. Hartmann, *Z. Chem.* **1971**, *11*, 344–345.
[15] I. N. Rosenberg, A. Goswami, *J. Biol. Chem.* **1979**, *254*, 12318–12325.
[16] S. Gnidehou, B. Caillou, M. Talbot, R. Ohayon, J. Kaniewski, M.-S. Noël-Hudson, S. Morand, D. Agnangji, A. Sezan, F. Courtin, A. Virion, C. Dupuy, *FASEB J.* **2004**, *18*, 1574–1576.
[17] I. N. Rosenberg, A. Goswami, *Methods Enzymol.* **1984**, *107*, 488–500.
[18] D. D. Brown, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 13001–13016.
[19] R. S. Talekar, G. S. Chen, S.-Y. Lai, J.-W. Chern, *J. Org. Chem.* **2005**, *70*, 8590–8593.
[20] E. Yeh, L. C. Blasiak, A. Koglin, C. L. Drennan, C. T. Walsh, *Biochemistry* **2007**, *46*, 1284–1292.
[21] A. Osborne, R. N. F. Thorneley, C. Abell, S. Bornemann, *J. Biol. Chem.* **2000**, *275*, 35825–35830.

Received: September 19, 2007
Published online on January 28, 2008

