Expression of a soluble form of iodotyrosine deiodinase for active site characterization by engineering the native membrane protein from *Mus musculus*

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Abstract: Reductive deiodination is critical for thyroid function and represents an unusual exception to the more common oxidative and hydrolytic mechanisms of dehalogenation in mammals. Studies on the reductive processes have been limited by a lack of convenient methods for heterologous expression of the appropriate proteins in large scale. The enzyme responsible for iodide salvage in the thyroid, iodotyrosine deiodinase, is now readily generated after engineering its gene from *Mus musculus*. High expression of a truncated derivative lacking the membrane domain at its N-terminal was observed in Sf9 cells, whereas expression in *Pichia pastoris* remained low despite codon optimization. Ultimately, the desired expression in *Escherichia coli* was achieved after replacing the two conserved Cys residues of the deiodinase with Ala and fusing the resulting protein to thioredoxin. This final construct provided abundant enzyme for crystallography and mutagenesis. Utility of the *E. coli* system was demonstrated by examining a set of active site residues critical for binding to the zwitterionic portion of substrate.

Keywords: heterologous expression; membrane anchor; mammalian; dehalogenase; protein engineering; deiodinase; iodotyrosine

Introduction

Recognition and processing of carbon-halogen bonds are central to the metabolism of numerous synthetic and natural compounds that are used in medicine and industry.^{1–5} Biological degradation of organic halides is typically accomplished under aerobic con-

ditions by oxidation or substitution reactions. Reductive dehalogenation is an alternative common to anaerobes but rarely detected in aerobes.^{6–9} Two exceptions are key to human health, and both relate to the synthesis and regulation of the thyroid hormone thyroxine (3,3',5,5'-tetraiodothyronine). Iodotyrosine deiodinase (IYD) promotes reductive deiodination of iodotyrosine formed as a byproduct of thyroxine biosynthesis [Eq. (1)].^{10–12} This enzyme is essential for recovering iodide for subsequent reuse in thyroxine production. Its mutation can lead to iodide deficiency and ultimately hypothyroidism.^{13,14} The other exception, iodothyronine deiodinase (ID), acts on thyroxine and its derivatives to potentiate their physiological activity [Eq. (2)].^{15,16} Deiodinating the outer ring of thyroxine enhances the cellular effects of the hormone. Conversely, deiodinating the inner ring suppresses the cellular effects.

Abbreviations: IYD, iodotyrosine deiodinase; ID, iodothyronine deiodinase; TRX, thioredoxin; MIT, moniodotyrosine; DIT, diiodotyrosine.

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Similarities between IYD and ID extend only to their common ability to dehalogenate substrates by reduction. IYD is a flavoprotein of the NADH oxidase/flavin reductase structural superfamily^{11,12} and utilizes NADPH as its physiological source of reducing equivalents.¹⁷ In contrast, ID contains an active site selenocysteine,¹⁸ belongs to the thioredoxin structural superfamily¹⁹ and utilizes the reducing power of thiols.^{20,21} Mechanistic investigations on these fascinating and unique enzymes have been thwarted by the lack of ready access to pure and abundant protein. The significance of IYD and ID in physiology, medicine, enzymology and even perhaps bioremediation has nonetheless inspired many attempts to develop convenient systems for their expression and isolation.

Initial difficulties in expression of these enzymes were attributed to their membrane association and mammalian origins. Cell culture remains the most common source of ID^{22,23} despite recent success in heterologous expression of ID in yeast.²⁴ Attempts to generate a soluble form of ID have not yet been successful. Deletion of its N-terminal membrane anchor yielded only inactive protein.²⁵ Strategies for preparing IYD did not fare much better than those for ID until the efforts described in this article. The first isolation of pure IYD was accomplished in very low yield (<5% initial activity recovered) and required detergent extraction of thyroid microsomes followed by precipitation and multiple chromatographic procedures.¹⁰ Limited proteolysis by trypsin was subsequently discovered to remove the N-terminal membrane anchor of IYD and release a soluble and active domain from thyroid microsomes.¹² However, neither the purity nor stability of this fragment was sufficient for mechanistic and structural studies.

Identification of the IYD gene allowed for its cloning and expression in mammalian cells.^{11,12} This

Figure 1. Iodotyrosine deiodinase is comprised of three protein domains: a transmembrane domain (residues 1–24), an inter-domain (residues 25–82), and a NADH oxidase/flavin reductase domain (residues 83–285). Excision of the N-terminal residues 2–33 removes the membrane anchor and results in a soluble derivative of the enzyme IYD(Δ tm).

system supported mutagenesis studies on IYD as long as only catalytic amounts of protein were necessary. Deletion of residues 2–33 also generated a soluble and stable form of the enzyme IYD(Δ tm) (Fig. 1).²⁶ Further truncation of IYD to its core domain homologous to the NADH oxidase/flavin reductase superfamily resulted in insoluble and inactive protein when expressed in HEK 293.²⁷ Fusion of this domain to glutathione S-transferase, maltose binding protein, (His)₆, NusA, or thioredoxin also yielded insoluble and inactive protein when expressed in *Escherichia coli*.²⁷ As described below, IYD(Δ tm) constructs have since been expressed in *Pichia pastoris* and Sf9 cells. Neither host was optimal for large-scale preparation or routine mutagenesis. Accordingly, further engineering of the IYD gene was initiated and ultimately provided reliable expression of a soluble form of IYD in *E. coli*. Subsequent crystallographic studies described in this report indicate that this new derivative shares an equivalent active site structure with a previous construct expressed in Sf9 cells. New opportunities for correlating enzyme structure and function are now possible and have been illustrated initially by mutation of key residues in the active site that coordinate to the zwitterionic region of substrate.

Results

Eukaryotic expression of IYD(Δ tm)

The methylotropic yeast *Pichia pastoris* was the first host of choice for deiodinase expression to overcome the folding problems previously encountered with *E. coli* and the limited material available from mammalian cell culture. The original IYD(Δ tm) gene contains codons that are very rarely encountered by *Pichia* according to the Codon Usage Database (Kazusa DNA Research Institute).²⁸ A synthetic gene of IYD(Δ tm) was consequently constructed to prevent overuse of rare codons in *Pichia* and minimize stable mRNA secondary structures that might interfere with expression (Fig. 2). Rare codons with a frequency of less than 5 per thousand in *Pichia* represented 2% of the *Mus musculus* IYD(Δ tm) gene. These were all substituted with codons of greater abundance in the host. Codons with a

M	S	A	Q	V	Q	P	W	V	D	E	D	L	K	D	S	T	E	D	L	20
ATG		GCT	CAA	GTT	CAG	CCC	TGG	GTG	GAT	GAA	GAC	TTG	AAA	GAC	AGC	ACA	GAA	GAC	CTT	
	TCT				<u>CCT</u>								AAG	GAT	<u>AGT</u>	ACC			TTA	
Q	V	E	E	D	A	E	E	W	Q	E	A	E	E	S	V	E	H	I	P	40
CAA	GTG	GAA	GAA	GAT	GCT	GAG	GAG	TGG	CAA	GAG	GCA	GAG	GAG	AGT	GTG	GAG	CAC	ATC	CCG	
				GCC	GAA				GAA		GAA		TCA	GTT	GAA	<u>CAT</u>	ATT	<u>CCA</u>		
F	S	H	T	R	Y	P	E	Q	E	M	R	M	R	S	Q	E	F	Y	E	60
TTC	TCT	CAC	ACC	CGA	TAC	CCA	GAA	CAG	GAA	ATG	AGG	ATG	AGG	TCC	CAG	GAA	TTC	TAT	GAG	
TTT	TCC		ACT	<u>AGA</u>	TAT			CAA	GAG		<u>AGA</u>		CGT	AGT			TTT	TAC		
L	L	N	K	R	R	S	V	R	F	I	S	S	E	H	V	P	M	E	V	80
CTC	CTC	AAT	AAG	AGA	CGC	TCC	GTC	AGG	TTT	ATC	AGC	AGC	GAG	CAC	GTC	CCA	ATG	GAA	GTC	
<u>TTG</u>	<u>CTT</u>	AAC	AAA		AGG	TCT	GTT	<u>AGA</u>	TTC	ATA	<u>TCA</u>	<u>TCC</u>	GAA	<u>CAT</u>	GTA	CCT				
I	E	N	V	I	K	A	A	G	T	A	P	S	G	A	H	T	E	P	W	100
ATT	GAA	AAT	GTC	ATC	AAA	GCA	GCA	GGA	ACA	GCT	CCA	AGT	GGG	GCC	CAT	ACA	GAG	CCC	TGG	
ATC	GAG		GTG	ATT	AAG	GCT		ACT			TCT	<u>GGT</u>			GAA	<u>CCT</u>				
T	F	V	V	V	K	D	P	D	M	K	H	K	I	R	E	I	I	E	E	120
ACC	TTT	GTG	GTG	GTG	AAG	GAC	CCA	GAC	ATG	AAG	CAT	AAG	ATC	AGA	GAG	ATT	ATC	GAA	GAG	
ACT		GTT	GTC	GTT	AAA	GAT				CAC	AAA					ATT				
E	E	E	I	N	Y	M	K	R	M	G	K	R	W	V	T	D	L	K	K	140
GAG	GAA	GAA	ATA	AAT	TAC	ATG	AAA	AGG	ATG	GGA	AAG	CGA	TGG	GTC	ACA	GAC	CTG	AAG	AAA	
GAA		GAG	ATC	AAC	TAT		AAG	<u>AGA</u>		GGT	AAA	CGT		GTG	ACT	GAT	TTG			
L	R	T	N	W	I	K	E	Y	L	D	T	A	P	V	L	I	L	I	F	160
CTG	AGA	ACC	AAC	TGG	ATT	AAG	GAG	TAC	TTG	GAC	ACC	GCC	CCA	GTT	CTG	ATC	CTC	ATT	TTC	
CTT		ACT	AAT			GAA				GCT			TTA		<u>CTG</u>					
K	Q	V	H	G	F	A	A	N	G	K	K	K	V	H	Y	Y	N	E	I	180
AAA	CAA	GTC	CAT	GGT	TTT	GCT	GCG	AAT	GGA	AAG	AAG	AAG	GTC	CAC	TAC	TAC	AAC	GAG	ATC	
	GTT		GGA			<u>GCA</u>	AAC	GGT		AAA				TAT	AAT		ATA			
S	V	S	I	A	<u>C/A</u>	G	L	L	L	A	A	L	Q	N	A	G	L	V	T	200
AGT	GTG	TCC	ATC	GCC	<u>TGT</u>	GGC	CTC	CTG	CTG	GCT	GCA	CTG	CAG	AAT	GCAGGG	CTA	GTG	ACG		
TCT	GTT	TCA		GCT	<u>GCA</u>	<u>GGT</u>	<u>TTG</u>	CTT	CTA		GCC	TTG	CAA	AAC	GCT	<u>GGT</u>	TTA	GTT	<u>ACT</u>	
V	T	T	T	P	L	N	<u>C/A</u>	G	P	R	L	R	V	L	L	G	R	P	S	220
GTC	ACT	ACC	ACT	CCC	CTC	AAC	<u>TGT</u>	GGT	CCT	CGT	CTG	AGG	GTG	CTC	CTG	GGCCGC	CCC	TCA		
ACA				<u>CCA</u>	<u>CTT</u>	AAT	<u>GCT</u>	GGG		<u>AGA</u>	TTG	<u>AGA</u>	GTT	<u>TTA</u>	TTG	<u>GGT</u>	AGG	<u>CCA</u>	<u>TCC</u>	
H	E	K	L	L	V	L	L	P	V	G	Y	P	S	R	D	A	T	V	P	240
CAT	GAG	AAG	CTG	TTG	GTG	CTA	CTT	CCT	GTGGGG	TAC	CCC	AGC	AGA	GAT	GCC	ACA	GTG	CCT		
	GAA		CTA		CTG		CCC	GTT	<u>GGT</u>		<u>CCT</u>	<u>TCT</u>				ACC	GTT	CCA		
D	L	K	R	K	A	L	D	Q	I	M	V	T	V	H	H	H	H	H		260
GAC	CTC	AAG	CGG	AAA	GCT	CTG	GAC	CAG	ATC	ATG	GTG	ACA	GTA	CAC	CAT	CAC	CAT	CAC	CAT	
	<u>TTG</u>	AAA	<u>AGA</u>	AAG	GCA	TTA	GAT		ATT		GTA	<u>GTC</u>								

Figure 2. Nucleotide sequences used for expression of IYD(Δ tm). The nucleotide sequence derived from *Mus musculus* as shown below the amino acid sequence was used for expression of IYD(Δ tm) in Sf9 cells. The synthetic gene for IYD(Δ tm) used for expression in *Pichia* contained numerous codon changes specified below the parent nucleotide sequence. The underlined sequences replace the original *Mus musculus* codons that had a frequency in *Pichia* of less than 5 per thousand. The only changes to the *Mus musculus* gene used for expression in *E. coli* are indicated within the two boxes and encoded the C217A;C239A mutations.

frequency of 5–10 per thousand in *Pichia* were also reduced from 13 to 5% of the *Mus musculus* gene. Repetitive use of GAG to encode six Glu within a stretch of 11 residues (from 27 to 37) was avoided by replacing some GAG codons with GAA (Fig. 2). Finally, the overall stability of the predicted secondary structure of the gene transcript was reduced by 30% through further substitution of codons as guided by mFold.²⁹ In particular, the potential for the mRNA to form a hairpin near the start codon was suppressed by codon changes since secondary structure in this region can severely limit translation.³⁰

Recombination and expression of IYD(Δ tm) in *Pichia* produced detectable deiodinase activity although the yield of enzyme was small (Fig. 3,

Lane 1). The ability of *Pichia* to grow to high-cell densities compensated for weak expression and provided more than ~2 mg of enzyme per liter of media (Table I). Purification of IYD(Δ tm) required only a single step of affinity chromatography after cell lysis. Spectroscopic analysis suggested a ratio of two FMN per native α_2 dimer.³¹ The kinetic constants for the purified enzyme were also similar to those of IYD(Δ tm) expressed in HEK 293 (Table II),²⁶ although an increase in K_M resulted in a k_{cat}/K_M that was approximately threefold less for the enzyme from *Pichia* than that from HEK 293.

In contrast to the results with *Pichia*, IYD(Δ tm) was expressed very efficiently in Sf9 insect cells utilizing the baculovirus expression vector system of the Bac-to-Bac® kit as described previously.³¹ Sf9

Figure 3. Denaturing PAGE analysis of iodotyrosine deiodinase expression and purification from *Pichia*, Sf9, and *E. coli*. Expression levels of IYD(Δ tm) were determined from whole cell lysates from *Pichia* (Lane 1) and Sf9 (Lane 3). A HiTrap (Ni^{2+}) affinity column was used to purify soluble enzyme from *Pichia* (Lane 2) and Sf9 (Lane 4). Expression of the thioredoxin- IYD(Δ tm)* fusion protein was measured similarly for whole cell lysates of *E. coli* (Lane 5) and further separated into insoluble and soluble fractions (Lanes 6 and 7, respectively) by centrifugation (20g for 1 h). Again, a HiTrap (Ni^{2+}) affinity column was used to purify the soluble fusion protein (Lane 8) and finally a Mono Q column was used to isolate IYD(Δ tm)* (Lane 9) after digestion of the fusion protein with enterokinase. Molecular weight standards are shown (lane M).

cells infected with recombinant baculovirus containing the *Mus musculus* gene encoding IYD(Δ tm) generated very high expression equaling almost 10% of total protein. Enzyme purification required use of a single affinity column and yielded more than 40 mg of the deiodinase per L of culture (Table I). The FMN cofactor to protein dimer ratio was two, identical to that observed for the deiodinase expressed in *Pichia*. The kinetic constants for deiodination were also similar for these enzymes. The k_{cat}/K_M value for the deiodinase expressed in Sf9 cells was experimentally equivalent to that observed for IYD(Δ tm) expressed in *Pichia* and ~50% less than the value observed for the deiodinase expressed in HEK 293 cells (Table II).

Table I. Isolated Yields of Iodotyrosine Deiodinase Derivatives^a

Expression system/gene	Cell pellet (mg/g)	Culture (mg/L)	Purity (%)
<i>Pichia</i> /IYD(Δ tm)	0.18 $\pm$ 0.03	2.2 $\pm$ 0.6	91 $\pm$ 5
Sf9/IYD(Δ tm)	3.98 $\pm$ 0.03	43 $\pm$ 8	99 $\pm$ 0.1
<i>E. coli</i> /IYD(Δ tm)*	2.6 $\pm$ 1.0	11 $\pm$ 2	81 $\pm$ 9

^a All measurements were performed in triplicate. Purity was calculated by densitometry using ImageQuant 5.2. Both major fragments of IYD(Δ tm)* released from the TRX fusion construct were pooled for this analysis (see Lane 9, Fig. 3).

Prokaryotic expression of IYD

Original attempts to express IYD in *E. coli* yielded only insoluble protein despite the various fusion proteins tested. The native oxidation state of two conserved Cys residues was not initially known and was considered a possible cause for misfolding and inclusion body formation.²⁷ Subsequent crystallographic analysis indicated that both Cys were reduced,³¹ but contrary to our original expectation, a C217A;C239A mutant of the deiodinase expressed in HEK 293 cells retained significant catalytic activity (IYD*, Table II).²⁶ Thus, expression of various IYD(Δ tm) constructs with the mutations C217A;C239A (IYD(Δ tm)*) was attempted in *E. coli*. Ultimately, soluble protein was expressed from a plasmid encoding an N-terminal thioredoxin (TRX) fusion and polyhistidine tags at the C-terminus and in the linker region between the TRX and IYD(Δ tm)* genes (Supporting Information Fig. S1). Use of other fusion proteins in place of TRX, removal of either His₆ sequence, or lack of Cys to Ala mutation resulted in expression of only insoluble protein.

The TRX-IYD(Δ tm)* fusion gene was highly expressed in *E. coli* to yield the corresponding protein as 46% of total cellular protein (Lane 5, Fig. 3), and the vast majority of this was soluble (Lanes 6 vs. 7, Fig. 3). Isolation of the desired deiodinase required two chromatographic steps. First, the

Table II. Catalytic Properties of Iodotyrosine Deiodinase (*Mus musculus*) Derivatives^a

Source	Enzyme	DIT ^a			MIT
		k_{cat} (min ⁻¹)	K_M (μ M)	k_{cat}/K_M (min ⁻¹ μ M ⁻¹)	K_D (μ M)
HEK293	IYD ^b	7.1 $\pm$ 0.9	8 $\pm$ 3	0.89 $\pm$ 0.4	—
HEK293	IYD (Δ tm) ^b	5.8 $\pm$ 0.6	6 $\pm$ 2	0.95 $\pm$ 0.3	—
HEK293	IYD* ^b	16 $\pm$ 2	42 $\pm$ 7	0.38 $\pm$ 0.08	—
<i>Pichia</i>	IYD (Δ tm)	6.9 $\pm$ 1.3	19 $\pm$ 3	0.36 $\pm$ 0.09	—
Sf9	IYD (Δ tm)	4.5 $\pm$ 0.7	9 $\pm$ 1	0.49 $\pm$ 0.09	0.09 $\pm$ 0.04 ^c
<i>E. coli</i>	IYD (Δ tm)*	9.3 $\pm$ 1.6	40 $\pm$ 5	0.23 $\pm$ 0.05	2 $\pm$ 0.2
<i>E. coli</i>	IYD (Δ tm)* Y157F	65 $\pm$ 16	440 $\pm$ 170	0.15 $\pm$ 0.07	40 $\pm$ 10
<i>E. coli</i>	IYD (Δ tm)* E153Q	—	—	—	>1000

^a Kinetic measurements based on deiodination were fit to Michaelis–Menten kinetics (See Supporting Information).

^b Data from HEK 293 expression were determined previously.²⁶

^c From McTamney and Rokita.⁴⁵

Figure 4. (A) IYD(Δ tm)* (green and purple, 3TNZ) is overlaid with IYD(Δ tm) (3GFD, in gray) and Cys residues in yellow spheres. (B) The active site of IYD(Δ tm)* is established by residues of both polypeptides (green and purple) in the α_2 dimer. Three amino acid side chains (Tyr157, Glu153, and Lys178) are shown explicitly to indicate their direct coordination with the substrate MIT and indirect interaction with the active site FMN.

fusion protein was isolated by affinity of the His₆ sequence and then digested with enterokinase. IYD(Δ tm)* was next separated from TRX using a MonoQ anion exchange column to yield over 10 mg enzyme/L of culture with an acceptable purity of greater than 80% (Table I). This preparation included a small fraction (27%) of deiodinase that had been further truncated by enterokinase at the N-terminus since the C-terminal His₆ was still detected by Western blotting. Collectively, the pooled fractions of deiodinase contained 1.9 FMN per native enzyme dimer. The k_{cat} value of this enzyme was slightly greater and its K_{M} value was two to more than fourfold greater than those of the other deiodinase derivatives (Table II). As expected, however, IYD(Δ tm)* exhibited catalytic constants most similar to those of the C217A;C239A mutant (IYD*) expressed in HEK 293 cells that also exhibited high K_{M} and k_{cat} values but a rather standard $k_{\text{cat}}/K_{\text{M}}$ value.²⁶

Structural determination of IYD(Δ tm)*

The crystal structures of IYD(Δ tm)* and its complex with MIT were characterized to ensure that the Cys-to-Ala substitutions did not significantly perturb the enzyme structure and would allow for subsequent investigations of the catalytic mechanism. Cys217 is located at the interface of the homodimer, and Cys239 is closer to the protein surface but still protected from solvent [Fig. 4(A)].³¹ IYD(Δ tm) and IYD(Δ tm)* both crystallized under similar conditions of 0.2 M ammonium acetate, 0.1 M BisTris (pH 6.5

and 5.5, respectively), and 45% v/v 2-methyl-2,4-pentanediol. The structures of IYD(Δ tm)* and IYD(Δ tm)*.MIT were solved by molecular replacement and remained very consistent with the parent structures (Table III). In the absence of MIT, the active site appeared very accessible to solvent due to a lack of detectable structure in two surrounding regions of the polypeptide. In the presence of MIT, an active site lid comprised of a helix and loop was detected from the diffraction data. This lid effectively sequesters the substrate-flavin complex from solvent. The prospective use of *E. coli* expressed IYD(Δ tm)*.MIT as a model of native enzyme was validated by the very low RMSD of 0.262 Å when overlaid with the original IYD(Δ tm).MIT (3GFD) structure [Fig. 4(A)]. The small deviations in folding are not localized but rather distributed throughout the three-dimensional structure.

Most importantly, the active site characteristics of IYD(Δ tm) and its C217A;C239A mutant are essentially identical. Their cocrystals both revealed the same overall contacts between the protein, FMN and MIT.³¹ The aromatic portion of MIT stacks over the isoalloxazine ring of FMN, and its phenolic —OH forms hydrogen bonds to the 2'-hydroxyl group of the FMN ribose and the Ala126 backbone nitrogen. The carbon of MIT bonded to the iodide is only slightly more distant from the C4a of FMN than that detected in the parent enzyme expressed in Sf9 (3.73 Å vs. 3.65 Å, respectively).³¹ The zwitterionic arm of MIT is bound through a network of polar interactions including hydrogen bonding to the N-3

Table III. Crystallographic Parameters of IYD(Δ tm)* and Its co-crystal With MIT

	IYD (Δ tm)*	IYD (Δ tm)*.MIT
PDB Code:	3TO0	3TNZ
Data Collection		
Space group	<i>P</i> 31	<i>P</i> 3
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	87.270, 87.270, 62.725	108.98, 108.98, 49.39
α , β , γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 120.00
Molecules/asymmetric unit	2	2
Wavelength (Å)	1.54	0.9795
Resolution (Å)	19.88-2.66	50-2.25
<i>R</i> _{sym} (last shell)	0.105	0.15
<i>I</i> / σ <i>I</i>	73	12.4
Completeness (%)	99.71	99.7
Redundancy	3.7	8
Refinement		
Resolution (Å)	28.566-2.655	26.5-2.248
<i>R</i> _{work} / <i>R</i> _{free} (%)	18.23/23.69	17.2/20.0
No. of protein residues per monomer	221/222	221/221
No. of nonprotein atoms		
Ligand	80	128
Solvent	50	298
Mean <i>B</i> -factors (Å) ²	6.14	10.05
RMS deviations		
Bond lengths (Å)	0.015	0.014
Bond angles (°)	1.639	1.384
Ramachandran plot		
Most favorable	94.96%	97.00%
Additionally allowed	4.76%	2.50%
Disallowed	0.28%	0.50%

and O⁴ of the flavin ring and the side chains of three amino acid residues (Glu153, Tyr157, Arg178) as part of the active site lid [Fig. 4(B)].

Mutation of active site residues that coordinate to the zwitterionic region of substrate

Engineering IYD for expression in *E. coli* not only simplifies its preparation but also expedites its mutagenesis. These advances were applied first to measure the individual contributions of the three residues above that were expected to be critical for stabilizing the active site lid and activating the flavin indirectly through the intervening ammonium and carboxylate groups. The most conservative substitutions E153Q, Y157F, and K178Q were generated individually by standard oligonucleotide-directed mutagenesis. The K178Q mutant of IYD(Δ tm)* expressed in *E. coli* but was insoluble and inactive. This was not pursued further. However, the two remaining mutants alternatively containing E153Q and Y157F were expressed in a soluble form. Lack of the phenolic —OH of Y157F increased the *k*_{cat} and *K*_M values for deiodination by more than sevenfold and decreased the *k*_{cat}/*K*_M value more modestly by less than 40% (Table II, Supporting Information Fig. S3). In contrast, neutralizing the charge of

Glu153 by substitution with Gln reduced the deiodinase activity to an undetectable level. Dissociation of FMN from the active site was not responsible for these changes since its occupancy remained constant at one per active site in these two mutant enzymes as well as the parent IYD(Δ tm)*. In addition, CD spectra of the deiodinase variants indicated no significant change in the extent of their α -helix or β -sheet structures (Supporting Information Fig. S5). Only the E153Q mutant exhibited some perturbation in its spectrum near 210 nm.

Active site binding of ligands was measured independently from enzyme turnover by use of the fluorescent properties of the bound flavin. The E153Q mutant exhibited no measurable binding affinity for MIT, which could in part explain its lack of catalytic activity (Table II, Supporting Information Fig. S4). The Y157F mutant weakened the binding of MIT as well but only by 20-fold relative to its IYD(Δ tm)* parent. Almost the same extent of binding affinity was also lost by the substitution of the two Cys for Ala despite their distance from the active site (Table II). These initial substitutions did not affect the CD spectrum in the visible region based on the flavin absorbance (Supporting Information Fig. S6). Both IYD(Δ tm) expressed in *Pichia* and IYD(Δ tm)* expressed in *E. coli* generated equivalent spectra in the absence of MIT and responded similarly to addition of MIT. Subsequent mutation of Y157F had minimal effect on its CD spectrum, but not surprisingly, mutation of E153Q dramatically diminished its response to MIT.

Discussion

Deiodination of iodotyrosine is critical for iodide homeostasis, and deiodination of thyroxine is critical for metabolic regulation. Together, these processes offer unique systems for identifying new catalytic mechanisms in nature, but investigations have floundered in the absence of convenient and large-scale methods for their expression. *E. coli* remains the host of choice and was initially expected to support production of mammalian IYD. This enzyme is a member of a structural superfamily that is well represented in a wide range of bacteria. Other members of this protein superfamily are easily expressed by *E. coli* in large scale,^{32–35} and neither the bacterial nor mammalian enzymes appear to require post-translational modification.^{12,33–36} However, native IYD is membrane bound due to its hydrophobic N-terminal sequence (Fig. 1).²⁶ Bacterial homologues lack this sequence and do not associate with membranes. Consequently, a mammalian gene for IYD was truncated to include just the domain that aligns with the superfamily of soluble homologues. Expression of this derivative in a Rosetta strain of *E. coli* produced only inclusion bodies as did various fusions described above in the introduction.²⁷ A more

modest truncation that merely deleted the membrane anchor of IYD also formed only inclusion bodies when expressed by *E. coli* although this construct had previously produced native, soluble IYD(Δ tm) in HEK 293 cells.²⁶

Pichia represents a practical alternative to HEK 293 cells and provided modest yields of the deiodinase using a codon optimized gene for IYD(Δ tm) (Figs. 2 and 3). Additional optimization of the *Pichia* system was halted as soon as the extraordinary yield of deiodinase was realized from expression of IYD(Δ tm) in Sf9 cells (Fig. 3). This later host provided ample quantities of protein for crystallographic studies.³¹ The resulting structural information that was made available by this success now inspires many new and detailed questions that could be addressed by site-directed mutagenesis. Unfortunately, this procedure is tedious when expression is limited to Sf9 cells due to the necessity of generating new baculovirus for each desired construct. *E. coli* expression presents an optimum alternative for preparing numerous variants in large scale.

The two conserved Cys (C217 and C239) within the NADH oxidase/flavin reductase superfamily domain of IYD had initially been thought essential for the catalytic mechanism.³⁷ Once catalysis was discovered to be independent of Cys,²⁶ efforts were reinitiated to express new mutants in *E. coli*. Improper oxidation or reduction of the thiol side chain of Cys often complicates expression of recombinant proteins in bacteria.^{38,39} Double mutation of C217A;C239A in IYD was necessary but not sufficient for *E. coli* to express active enzyme. Subsequent removal of the *N*-terminal membrane anchor and addition of a *C*-terminal His₆ to form IYD(Δ tm)* in pET21a was also not sufficient for producing active enzyme. However, insertion of this gene into pET32a resulted in high expression of a soluble fusion protein formed by thioredoxin and the deiodinase flanked by His₆ on both C- and N-termini (Supporting Information Figs. S1 and S3). A comparable construct containing the native C217 and C239 formed only inclusion bodies.

The soluble double mutant IYD(Δ tm)* was readily available after removal of the fused thioredoxin and standard anion exchange chromatography. The resulting deiodinase is stable for over 1 month at 4°C and exhibits kinetic constants sufficiently similar to the native enzyme (Table III). Crystallographic studies also reveal no significant differences between IYD(Δ tm)* and IYD(Δ tm). Thus, the variant expressed in *E. coli* is suitable for further mutagenesis to identify the structural and chemical origins of catalyzing reductive dehalogenation under aerobic conditions.

Numerous active site residues are likely responsible for catalysis and binding of substrate and FMN. Previous studies identified key amino acids

involved in FMN binding by sequencing IYD from humans that had thyroid disease caused by defects in this enzyme.¹⁴ Later crystallographic studies identified Glu153, Tyr157, and Arg178 as key to substrate recognition.³¹ These residues may also be involved in positioning the active site lid and activating FMN for catalysis. Dissecting their role was now possible by expression of the deiodinase in *E. coli* for routine site-directed mutagenesis. Turnover kinetics and equilibrium binding performed to date demonstrate an absolute requirement of Glu153 for substrate binding. This result was not anticipated since only a single charge-charge interaction is removed by the E153Q substitution. Perhaps a fine balance is maintained between dynamic and fixed states of the lid region. In contrast, Tyr157 is not essential and contributes only modestly to substrate binding. The Y157F mutant expressed weak binding for MIT yet the k_{cat} increased as if the loss of affinity might result in faster release of substrate as well as product tyrosine during catalytic turnover. More detailed analysis of this and other mutants awaits future study.

Efforts to develop a convenient source for IYD progressed full circle through various hosts but began and finally ended with successful expression of soluble enzyme in high yield from *E. coli*. The generality of replacing native but nonessential Cys residues with Ala or other inert residues to improve bacterial expression of heterologous proteins is not yet known but worth further exploration.^{40,41} For IYD in particular, such mutation has provided a derivative that is now suitable for extensive study on the origins of its unusual activity.

Materials and Methods

Materials

Oligodeoxynucleotide primers were obtained from Integrated DNA Technology (Coralville, IA). All enzymes were purchased from New England Biolabs (Ipswich, MA). The plasmid pcDNA3.1(+)-IYD containing the gene for iodotyrosine deiodinase was constructed from wild-type *Mus musculus* IYD cDNA (I.M.A.G.E. clone 5061638) from ATCC (Manassas, VA) as described previously.²⁶ A synthetic variant of this gene flanked by EcoRI and NotI sites and inserted in pUCminusMCS was obtained from Blue Heron Biotechnology (Bothell, WA). All cell lines other than Rosetta 2(DE3) *Escherichia coli* (Novagen, San Diego, CA) were purchased from Invitrogen (Carlsbad, CA). Sodium [¹²⁵I]-iodide used for radiolabeling diiodotyrosine (DIT) was obtained from Perkin Elmer (Waltham, MA).⁴² Antibodies for Western blotting were purchased from Novagen. All other reagents were obtained at the highest grade available and used without further purification.

General methods

DNA isolation was performed using either a Qiaprep Mini Kit (Qiagen, Valencia, CA) or a GeneJet Plasmid Miniprep Kit (Fermentas, Glen Burnie, MD). PCR reactions were performed using an Eppendorf Mastercycler (New York, NY). Agarose gel electrophoresis (horizontal) was performed according to Ausubel⁴³ using 125 V and a standard of Mass Mix DNA Ladder (Fermentas). Ligations and dephosphorylations were performed under standard conditions using T4 ligase, and Antarctic phosphatase, respectively. *E. coli* transformations were performed according to Ausubel⁴³ with an Eppendorf Electroporator 2510 (1700 V, 1 mm gap cuvette), and samples were selected on LB plates containing ampicillin. DNA sequencing was performed by Geneway Research (Hayward, CA) and the University of Maryland Biotechnology Institute (College Park, MD). Cloning and expression of IYD in *Pichia pastoris* followed protocols provided with the EasySelect *Pichia* Expression kit (Invitrogen, Carlsbad, CA). Protein expression in Sf9 cells followed protocols provided with the Bac-to-Bac® Baculovirus Expression kit as described previously.³¹

Discontinuous SDS-PAGE gels (12 % acrylamide resolving and 5 % stacking) and Laemmli running buffer were prepared according to standard procedures⁴⁴ and run according to Ausubel⁴³ using 200 V and a Mini Protean 3 gel system (Bio-Rad, Hercules, CA). All protein gels were stained with Coomassie Brilliant Blue. Electrophoretic transfer of proteins from SDS-PAGE gels to PVDF membranes (Invitrogen) for western blotting was performed with a Bio-Rad Mini Trans-Blot Cell according to the manufacturer's directions. Western Blot recognition was performed according to Ausubel⁴³ using an anti-His-Tag monoclonal antibody, a goat anti-mouse IgG alkaline phosphatase conjugate, and ECF (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

UV measurements were obtained with a Hewlett-Packard 8453 spectrophotometer (Palo Alto, CA). CD measurements were made on a Jasco J-810 spectropolarimeter (Easton, MD). Ten spectra were collected at 20°C in a 0.1-cm pathlength quartz cuvette. Ligand binding was monitored by the change in bound flavin fluorescence using λ_{ex} of 450 nm and λ_{em} of 527 nm as reported previously.⁴⁵ Catalytic deiodination of [¹²⁵I]-DIT was determined by discontinuous measurement of [¹²⁵I]-iodide release described previously using DIT concentrations ranging from 1.0 to 50 μM and dithionite as the reductant (1 % w/v).^{12,42} Assays were performed in triplicate and data were fit to Michaelis-Menton kinetics using Origin 7.0 (Northampton, MA).

Plasmid construction and host transformation

The codon optimized gene for IYD (*Mus musculus*) with truncation of its transmembrane domain (resi-

dues 2–33) and addition of a C-terminal His-tag (IYD(Δtm), see Fig. 2) was synthesized by Blue Heron Technologies. This construct was then excised from its pUC19(–) derivative and ligated into pPICZa after EcoRI and NotI digestion to yield pPICZa-optIYD(Δtm). Ligated plasmids were transformed into One Shot Top10 cells. Clones for pPICZa-optIYD(Δtm) were identified by sequencing DNA from colonies demonstrating resistance to ampicillin. Plasmid pPICZa-optIYD(Δtm) was linearized with BstXI for recombination into electrocompetent histidine deficient GS115 *Pichia pastoris* cells.⁴⁶ *Pichia* colonies exhibiting zeocin resistance were analyzed for the presence of the IYD gene by adapting the Easy Select Kit protocol for direct analysis of cells by colony PCR. The colonies were suspended in water to an OD₆₀₀ of 20, heated in a microwave set at high for 1 min, and then directly amplified by PCR in the presence of 20 μM primers supplied from the manufacturer. PCR products of the expected size were purified by gel electrophoresis, and their identity was confirmed by DNA sequencing. Transformants containing the gene for IYD were also screened for a phenotype of methanol utilization (Mut⁺).

For transforming *E. coli* with the deiodinase, a truncated and His-tagged variant of the IYD gene containing C217A; C239A (IYD(Δtm)*) was amplified using a template based on pcDNA3.1(+) constructed previously²⁶ and primers 5'-AAGCTTAAGCTTGGATCCGCCACCATGGCTCAAGTTCAGCCC-3' and 5'-CTCGAGCTCGAGCTAATGGTGTATGGTGTATGGTGTATCTCACCATGATC-3'. The resulting PCR product and pET32a were digested with BamHI and XhoI, ligated together and used to transform One Shot Top10 cells. DNA was extracted from colonies resistant to ampicillin and sequenced to confirm the presence of the thioredoxin (TRX) fusion pET32a-TRX-IYD(Δtm)*. This plasmid was then used to transform electrocompetent Rosetta 2(DE3) *E. coli*, and colonies were selected for ampicillin resistance.

Active site mutations to the gene were generated by site-directed mutagenesis of the pET32a plasmid encoding TRX-IYD(Δtm)*. E153Q was introduced by the forward primer 5'-GAAGAGGAGCAA GAAATTAATTACATGAAAAGGATGGGAAAGCGAT GGG-3' and its reverse complement. Y157F was created by the forward primer 5'-GAGGAGGAAGAAAT AAATTTTCATGAAAAGGATGGGAAAGCGATGGG-3' and its reverse complement. K178Q was formed by using the forward primer 5'-AGAACCAACTGGATT CAGGAGTACTTGGACACCGCCCCAGTTCTGATCC T-3' and its reverse complement. The mutated codon is underlined, and the nucleotide substitution used for the amino acid change is noted in bold. The desired mutation was confirmed by sequencing, and the plasmids were used to transform electrocompetent Rosetta 2(DE3) *E. coli* for expression.

Expression of IYD

Pichia containing IYD(Δ tm) with a Mut⁺ phenotype was grown in minimal glycerol media plus histidine to an OD₆₀₀ of 2–6 (30°C) and then diluted to an OD₆₀₀ of 1 with minimal methanol media plus histidine (MMH) for induction. Cells were grown in this media for 48 h, isolated by centrifugation, and resuspended for subsequent affinity purification (500 mM sodium chloride, 50 mM sodium phosphate pH 8, 10 mM imidazole, 150 μ M FMN). Cells were lysed by three passages through a French press at approximately 10,000 psi. Lysates were centrifuged at 12,000g for 10 min (4°C) to remove cellular debris. The resulting supernatant was centrifuged a second time at 20,000g for 1 h (4°C) and finally filtered (0.22 μ m) to remove any additional particulate matter.

Expression of deiodinase in Sf9 cells was described previously.³¹ For expression in *E. coli*, Rosetta 2(DE3) clones containing pET32a-TRX-IYD(Δ tm)* were grown in LB media with ampicillin and chloramphenicol at 37°C up to an OD₆₀₀ of 0.5. The medium was then cooled to 18°C (30 min), induced by addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside and incubated with shaking for 4 h (18°C). Cells were harvested and prepared for affinity purification as described earlier.

Affinity purification of IYD

IYD(Δ tm) was purified on a HiTrap HP column (1 mL) chelated with Ni²⁺ using an AKTA FPLC (GE Healthcare Bio-Sciences Corp.). Soluble lysates of *Pichia* and Sf9 cells were applied to the affinity column, washed with 5 column volumes of wash buffer (500 mM sodium chloride, 50 mM sodium phosphate pH 8.0, 20 mM imidazole) and eluted with a linear gradient of 20–300 mM imidazole in wash buffer (20 mL). Fractions containing the deiodinase were identified by SDS-PAGE and then pooled and dialyzed against 10 mM potassium phosphate pH 7.4. Lysates of *E. coli* were treated similarly to those above except the pooled fractions were dialyzed against 0.2 M Tris-HCl pH 7.4. Enterokinase was then added to the dialyzed protein, and the resulting solution was incubated for 16 h at 18°C. IYD(Δ tm)* was separated from TRX by anion exchange (Mono Q, GE Healthcare Bio-Sciences Corp) using a wash of five-column volumes of 0.2 M Tris-HCl pH 7.4 and elution with a linear gradient of 0–1 M NaCl in wash buffer (20 mL). Active site mutants of this protein were also purified in the same manner.

The concentration of enzyme-bound FMN was determined by A₄₅₀ (ϵ ₄₅₀ 12,500 M⁻¹ cm⁻¹),⁴⁷ and the concentration of deiodinase was likewise determined by A₂₈₀ using an extinction coefficient of 38,000 M⁻¹ cm⁻¹ as determined by the Edelhoch method using enzyme purified from Sf9 cells.⁴⁸

Crystallography of IYD(Δ tm)*

IYD(Δ tm)* was crystallized by the hanging drop diffusion method using one part IYD (10 mg/mL; 10 mM potassium phosphate, pH 7.4) to one part reservoir solution containing 0.2 M ammonium acetate, 0.1 M BisTris (pH 6.5), and 45% v/v 2-methyl-2,4-pentanediol. IYD(Δ tm)*-MIT co-crystals were formed at 20°C in 15% w/v PEG 10,000, 20% glycerol, 0.1 M citric acid (pH 5.5). Diffraction data for IYD(Δ tm)* were collected at 100 K using a Bruker (Billerica, MA) Microstar H2 generator with Proteum Pt135 CCD detector at a wavelength of 1.54178 Å. Diffraction data for IYD(Δ tm)*-MIT were collected at 100 K from the Advanced Photon Source with a CCD detector at a wavelength of 0.9795 Å. Data were integrated and scaled using Proteum. Molecular replacement was performed by PHASER in the CCP4 program suite. The structures were refined by iterations in COOT. Refinement statistics are listed in Table III.

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