

Single Amino Acid Switch between a Flavin-Dependent Dehalogenase and Nitroreductase

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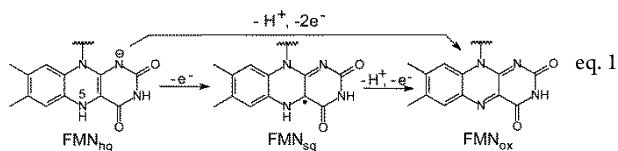
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S Supporting Information

ABSTRACT: A single mutation within a flavoprotein is capable of switching the catalytic activity of a dehalogenase into a nitroreductase. This change in function correlates with a destabilization of the one-electron-reduced flavin semiquinone that is differentially expressed in the nitro-FMN reductase superfamily during redox cycling. The diversity of function within such a superfamily therefore has the potential to arise from rapid evolution, and its members should provide a convenient basis for developing new catalysts with an altered specificity of choice.

Enzymes capable of reducing nitro groups are key to a wide range of applications, including bioremediation,¹ fine chemical production,² and drug activation.³ One class of nitroreductases (NRs) promotes single-electron transfer and exhibits sensitivity to oxygen. This latter property has generally limited their utility, despite success in detecting hard tumors and activating prodrugs in vivo.⁴ An alternative class of NRs are oxygen-insensitive based on their ability to promote hydride transfer and suppress single-electron processes. This class provides most opportunities in industry and medicine and has inspired the search for new NRs by genomic sequencing.⁵ Considerable effort has also been devoted to engineer existing NRs⁶ for optimizing their regioselectivity, catalytic efficiency, and stability. NRs have additionally been engineered from a nontraditional source^{6b} but not from enzymes that share a similar architecture yet diverge in catalytic function. The latter strategy has now generated a new NR by substitution of only a single amino acid.

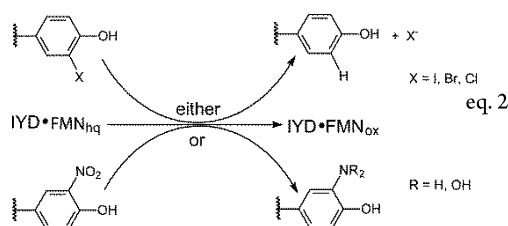
The majority of oxygen-insensitive NRs belong to a structural superfamily of flavoproteins entitled nitro-FMN reductases.^{1c} These proteins share an ability to destabilize the one-electron ($1e^-$)-reduced flavin semiquinone (FMN_{sq}) and consequently inhibit single-electron processes.⁷ Not even a trace of the FMN_{sq} (<0.03%) was detected after careful titration of an NR from its oxidized form (FMN_{ox}) to its two-electron ($2e^-$)-reduced hydroquinone (FMN_{hq}) (eq 1). Recently,



another branch of this superfamily has been identified. This includes enzymes with two quite divergent activities, and both

likely require stabilization of FMN_{sq} .⁸ One has been entitled a flavin destructase (BluB) for its O_2 -dependent conversion of FMN into 5,6-dimethylbenzimidazole.⁹ The second, entitled iodotyrosine deiodinase (IYD), catalyzes reductive dehalogenation of halotyrosines.^{10,11} In contrast to NR, BluB and IYD do not utilize NAD(P)H directly but rather require a separate reductase to generate their FMN_{hq} in vivo.^{9,12} Since the reductase for IYD has not yet been identified, dithionite has become the reductant of choice for the majority of studies, including those below.

An empirical correlation emerges for the nitro-FMN reductase superfamily with regard to catalytic function and the type of hydrogen bonding available to the N5 position of the bound FMN. NR provides an amide NH for interacting with the FMN N5, whereas IYD and BluB provide a side-chain OH from Thr or Ser. Due to the importance of the N5 position in the redox chemistry of FMN,¹³ this dichotomy had the potential to predict the redox chemistry as well. Thus, IYD became an interesting candidate for generating an NR by changing a single hydrogen-bonding partner (eq 2). IYD was also appealing since an early survey of inhibitors suggested that both nitro- and dinitrotyrosine likely bind tightly to the active site of IYD.¹⁴



IYD was first discovered in humans while the biochemical origins of thyroid disease were being investigated.¹⁵ This enzyme has since been identified in numerous metazoa and certain bacteria but not plants, fungi, or protozoa.¹¹ Native IYD from bacteria lack a membrane anchor common to the mammalian enzyme, and heterologous expression of the gene from *Haliscomenobacter hydrophila* has produced the most robust deiodinase (hhIYD) to date.¹¹ This represents a particularly appealing target for environmental engineering since *H. hydrophila* can be found in sewage treatment plants.¹⁶ Wild-type (wt) hhIYD has been expressed again as a control for the studies described below. The rate constants for deiodination of

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Table 1. Catalytic Properties of wt hhIYD and Its T173A Mutant

protein	I ₂ -Tyr				O ₂ N-Tyr
	<i>k</i> _{cat} (min ⁻¹)	<i>K</i> _M (μM)	<i>k</i> _{cat} / <i>K</i> _M (μM ⁻¹ min ⁻¹)	<i>K</i> _D (μM)	<i>K</i> _D (μM)
wt hhIYD	14 ± 2	4 ± 1	3 ± 1	2.9 ± 0.1	17 ± 2
T173A hhIYD	5.6 ± 0.7	35 ± 8	0.2 ± 0.04	2.6 ± 0.2	5.3 ± 0.7

diiodotyrosine (I₂-Tyr) are similar to those determined previously (Table 1, Figure S2),¹¹ and I₂-Tyr binds wt hhIYD with high affinity, as measured by a standard assay based on quenching the fluorescence of the active-site FMN_{ox}.¹⁷ This same fluorescence assay has now confirmed that nitrotyrosine (O₂N-Tyr) strongly associates with wt hhIYD, although its *K*_D is 6-fold higher than that of I₂-Tyr (Table 1, Figure S1). If O₂N-Tyr adopts the same orientation as I-Tyr in the active site of IYD, then the nitro group will align with the FMN in analogy to its position in NRs.¹⁸

The basal ability of wt hhIYD to promote reduction of a nitro group was initially surveyed by the propensity of O₂N-Tyr to discharge the reducing equivalents of enzyme-bound FMN_{hq}. This same strategy had already been applied successfully when first exploring the dechlorinase and debrominase activity of mouse IYD.⁶⁷ Stoichiometric addition of dithionite to wt hhIYD under anaerobic conditions reduced its FMN_{ox} to FMN_{hq} as evident from the dramatic loss of absorbance at 450 nm (Figure 1A). Subsequently, the FMN_{ox} spectrum was

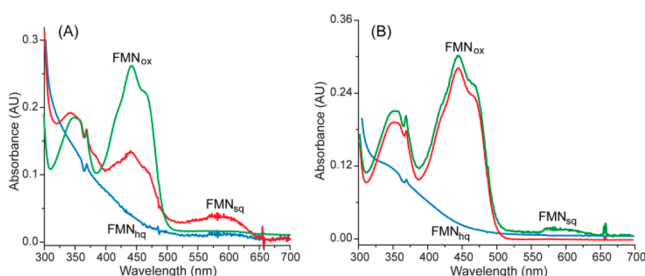


Figure 1. Oxidation and reduction of FMN within wt hhIYD and its T173A mutant. (A) hhIYD (wt, 18 μM) in NaCl (500 mM), 10% glycerol, and MES (100 mM, pH 6) containing its FMN_{ox} was fully reduced to its FMN_{hq} form (blue line) by stoichiometric addition of dithionite under anaerobic conditions. The FMN_{ox} signal (green line) was restored after incubation with I₂-TYR (10 μM) for 30 min. Alternative incubation with O₂N-Tyr (8 μM) generated a spectrum (red line) consistent with a mixture of FMN_{sq} and FMN_{ox} that remained unchanged after 30 min. (B) T173A hhIYD (18 μM) in NaCl (500 mM), 10% glycerol and MES (100 mM, pH 6) was fully reduced to its FMN_{hq} form (blue line) by stoichiometric addition of dithionite under anaerobic conditions. Subsequent addition of I₂-TYR (10 μM) restored the FMN_{ox} signal (green line) after incubation for 30 min. Alternative addition of O₂N-Tyr (8 μM) also restored the FMN_{ox} signal (red line).

restored almost completely by addition of 10 μM I₂-Tyr. Only a minimal excess of C-I bonds (20 μM) was required for this oxidation of the FMN_{hq}-containing wt hhIYD (18 μM). In contrast, treatment of the reduced FMN_{hq} form of wt hhIYD with O₂N-Tyr did not restore the full FMN_{ox} spectrum and instead generated a signal derived primarily from the neutral FMN_{sq} (Figure 1A, eq 1).¹⁹ Thus, only limited reduction of O₂N-Tyr was possible before wt hhIYD stalled in a non-productive and partially oxidized form. This effect was observed using a substoichiometric concentration of O₂N-Tyr (8 μM), and even then, the majority of O₂N-Tyr remained unchanged

after extensive incubation (see below). Previously, only trace quantities of the FMN_{sq} had been detected with mouse and human IYD during turnover of Cl-, Br-, and I-Tyr.^{17,20} In contrast, the inert analogue F-Tyr dramatically stabilized the 1e⁻-reduced FMN_{sq} intermediate during redox titration of human IYD.⁸ The bacterial enzyme wt hhIYD is now shown to act similarly and readily stabilizes its bound FMN_{sq} in the presence of F-Tyr during redox titration with xanthine and xanthine oxidase (Figure 2A).

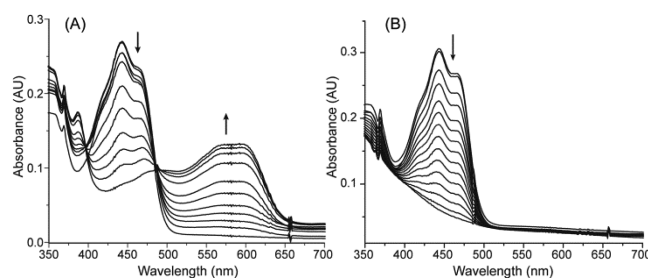


Figure 2. Redox titration of wt hhIYD and its T173A mutant in the presence of the substrate analogue F-Tyr. (A) FMN_{ox} of hhIYD was reduced by the xanthine/xanthine oxidase method²⁵ under anaerobic conditions in the presence of F-Tyr (500 μM). Arrows indicate consumption of FMN_{ox} (450 nm) and accumulation of FMN_{sq} (550–625 nm). (B) FMN_{ox} of the T173A mutant was reduced equivalently under anaerobic conditions in the presence of F-Tyr (500 μM). The arrow indicates the loss of absorbance at 450 nm associated with FMN_{ox} during its reduction to FMN_{hq}.

The lack of full transfer of electrons from FMN_{hq} to O₂N-Tyr implies that, at least for the nitro-FMN reductase superfamily, reduction of the nitro group predominantly relies on a hydride pathway. This in turn suggests that IYD's stabilization of FMN_{sq} could be preventing its full discharge of electrons to O₂N-Tyr. Consequently, destabilization of FMN_{sq} had the potential to unmask an efficient NR activity of IYD. Such destabilization is already a noted feature of NRs,⁷ and the correlation between catalytic activity and hydrogen bonding to the flavin N5 made this region an obvious target for manipulation. For IYD, hydrogen bonding between a Thr side-chain OH and the N5 position forms only after a halotyrosine binds to the active site and coincident with stabilization of its FMN_{sq} intermediate (Figure 2A).⁸ Mutation of the Thr to an Ala (T173A) in hhIYD eliminates this hydrogen bond. Such mutation did not affect binding of I₂-Tyr (Table 1), as anticipated from the lack of direct contact evident between the substrate and Thr173 in the crystal structure of mouse IYD.²¹ However, this residue does contribute to the catalytic efficiency of dehalogenation since the *k*_{cat}/*K*_M for the mutant decreases 15-fold relative to that of wt hhIYD (Table 1). An analogous Ser-to-Gly mutation in BluB similarly diminishes its catalytic activity by 30-fold.⁹

The T173A mutant of hhIYD gained an unexpected 3-fold increase in its affinity for O₂N-Tyr relative to that of wt hhIYD (Table 1). This is not likely caused by the loss of hydrogen

bonding to the N5 of FMN and instead may reflect a general easing of the steric constraints within the active site to accommodate the relatively bulky nitro group. Most importantly, O₂N-Tyr (8 μM) fully oxidize the FMN_{hq} form of the T173A mutant (18 μM) (Figure 1B). I₂-Tyr (10 μM) also oxidized this form of the mutant but yielded a trace of the FMN_{sq} (<10%) as well. This single mutation alone is consequently sufficient to limit formation of the FMN_{sq} intermediate and allow full discharge of the reducing equivalents of FMN_{hq} to the nitro compound. The lack of FMN_{sq} stabilization by the T173A mutant was similarly evident during its redox titration in the presence of F-Tyr. No FMN_{sq} was detected in stark contrast to the results with wt hhIYD (Figures 2). These data reinforce the empirical correlation between 1e⁻ vs 2e⁻ transfer processes, dehalogenation vs nitro reduction and hydrogen bonding to FMN N5.

Substoichiometric quantities of O₂N-Tyr were sufficient to oxidize FMN_{hq} completely in the T173A mutant. This is rather common for NRs, since the initial 2e⁻ reduction of nitro to nitroso is rarely detected and nitrosotyrosine in particular is highly unstable.^{5a,22} Most typically, the nitroso intermediate rapidly consumes an additional 2e⁻ from NR to form the corresponding hydroxylamine derivative.⁶ Less common but highly desired is the ability of NR to promote full reduction of a nitro compound to its amine derivative.^{5b}

As a complement to the initial spectrophotometric analysis used to monitor FMN_{hq} oxidation in hhIYD, consumption of O₂N-Tyr was monitored by reverse-phase HPLC (Figure S3). Reaction of O₂N-Tyr with the FMN_{hq} form of either wt or its T173A mutant hhIYD practically ceased after 10 min under ambient conditions in the absence of molecular oxygen (Figure 3). As anticipated from the spectral studies illustrated by

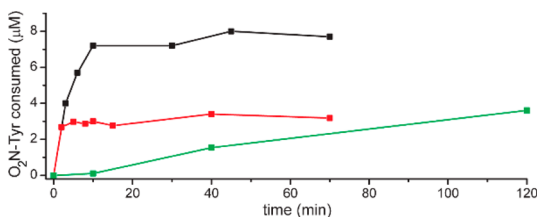


Figure 3. Reduction of O₂N-Tyr by enzyme-bound and free FMN_{hq}. Consumption of O₂N-Tyr (8 μM) by alternative addition of the reduced form (18 μM) of wt hhIYD (red points and line), its T173A mutant IYD (black points and line), and free FMN_{hq} (green points and line) under anaerobic conditions was monitored over time by reverse-phase HPLC (see Figure S3).

Figures 1 and 2, only approximately 35% of the total O₂N-Tyr (8 μM) was consumed by 18 μM of the reduced form of the wt hhIYD, whereas 100% of O₂N-Tyr (8 μM) was consumed by 18 μM of the equivalent T173A mutant. The initial rate of these reactions promoted by both enzymes was also considerably faster than that by free FMN_{hq} under equivalent conditions.

Incubation mixtures equivalent to those of Figure 3 were likewise treated with dansyl chloride to ensure retention of the possible tyrosine products on a reverse-phase column. Only one species was observed after O₂N-Tyr reduction with the T173A mutant, and its elution time corresponded to the conjugate of L-3-amino-tyrosine (H₂N-Tyr) containing two dansyl groups (Figure 4A,C). Coupling by two dansyl groups is typical of tyrosine,²³ and the identity of the didansylated

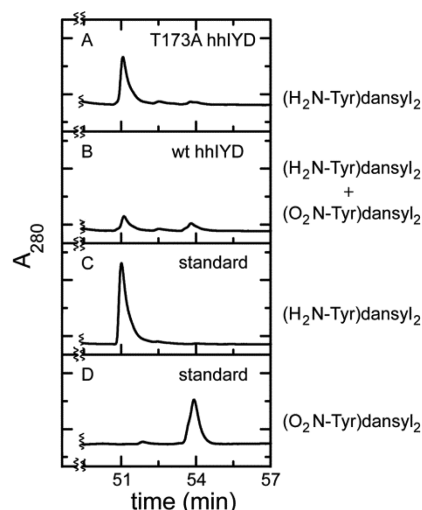


Figure 4. Detection of the O₂N-Tyr reduction product after derivatization by dansyl chloride. O₂N-Tyr (8 μM) was alternatively treated under anaerobic conditions for 50 min with (A) the reduced mutant T173A (18 μM) and (B) the reduced wt hhIYD (18 μM). (C) A standard of H₂N-Tyr. (D) A standard of O₂N-Tyr. Each sample and standard was ultimately treated with dansyl chloride (500 μM) before separation on reverse-phase C-18 HPLC using a gradient of solvent A (25 mM ammonium formate pH 5.7 and 14% acetonitrile) and B (acetonitrile) according to 0% B for 0–5 min; 0–8% B from 5 to 10 min; 8% B from 10 to 25 min; 8–45% B from 25 to 55 min; 45–58% B from 55 to 60 min (1 mL/min).

derivative was further confirmed by mass spectrometry (Figure S4). Low yields of the (H₂N-Tyr)dansyl₂ and (O₂N-Tyr)dansyl₂ were observed after reaction with the wt hhIYD (Figure 4B), consistent with the partial turnover of wt hhIYD as first revealed by UV–vis spectroscopy (Figure 1A).

Full reduction of O₂N-Tyr to H₂N-Tyr was not initially expected by the T173A mutant since NRs from the same superfamily do not typically provide sufficient reducing potential for this transformation.⁷ The stoichiometry of O₂N-Tyr reduction to H₂N-Tyr by FMN_{hq} was also not consistent with contributions from the enzyme alone. Three equivalents of enzyme-bound FMN_{hq} would have been consumed if it were solely responsible for reducing O₂N-Tyr to H₂N-Tyr. Instead, slightly more than 2 equiv of the reduced T173A mutant (18 μM) was consumed by O₂N-Tyr (8 μM) reduction, as measured by UV–vis spectroscopy (Figure 1B). Subsequent HPLC analysis confirmed this same stoichiometry (Figure S5). The additional reducing equivalents necessary to form H₂N-Tyr likely derive from the bisulfite that is generated during dithionite oxidation.²⁴ This byproduct (500 μM) does not react with O₂N-Tyr (8 μM) under anaerobic conditions comparable to those used for enzyme reaction (Figure S6) but does reduce a model intermediate, phenylhydroxylamine (8 μM), to aniline under equivalent conditions (Figure S6).

Multiple turnovers of reductive dehalogenation can be measured for IYD using excess dithionite since the aryl halide bond is stable to this source of reducing equivalents. In contrast, the nitro group is reduced spontaneously by dithionite, and thus the NR activity of IYD was initially measured in the absence of excess dithionite by single turnover of its FMN_{hq} to FMN_{ox} forms. However, the T173A mutant is capable of multiple turnovers as an NR. Sequential addition of dithionite and O₂N-Tyr for three cycles demonstrated full reduction and subsequent oxidation of its FMN (19 μM) and

full consumption of three additions of O₂N-Tyr (9 μM each) (Figure S7). Continuous turnover of the T173A mutant will also be possible once its native reductase or an appropriate surrogate is discovered. Efforts to find this reductase are ongoing and driven in part by its significance to iodide homeostasis in vertebrates.¹⁰

Only a single mutation of T173A endows the dehalogenase IYD with a new ability to catalyze reduction of a nitroaromatic substrate. This amino acid substitution is sufficient to alter hydrogen bonding to the N5 position of FMN and switch its ability to promote one- versus two-electron transfer processes. A correlation between catalytic function and hydrogen bonding at this position was evident in the nitro-FMN reductase superfamily but not previously recognized as a primary control of the catalytic chemistry. The results of this investigation illustrate how functional diversity within an enzyme superfamily may arise rapidly during evolution. In addition, the mutant T173A now offers a new platform to engineer unique NRs for in vivo and in vitro application as a complement to those efforts currently based on native NRs.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b07540.

Enzyme expression, purification, and assays; dansylation of enzyme products; phenylhydroxylamine reduction by bisulfite (PDF)

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■ Notes

The authors declare no competing financial interest.

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