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

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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, fine chemical production, and drug activation. 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. 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. Considerable effort has also been devoted to engineer existing NRs for optimizing their regiospecificity, catalytic efficiency, and stability. NRs have additionally been engineered from a nontraditional source 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. These proteins share an ability to destabilize the one-electron (1e\textsuperscript{-})-reduced flavin semiquinone (FMN\textsubscript{sq}) and consequently inhibit single-electron processes. Not even a trace of the FMN\textsubscript{sq} (<0.03%) was detected after careful titration of an NR from its oxidized form (FMN\textsubscript{ox}) to its two-electron (2e\textsuperscript{-})-reduced hydroquinone (FMN\textsubscript{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\textsubscript{sq}. One has been entitled a flavin deconstructase (BluB) for its O\textsubscript{2}-dependent conversion of FMN into 5,6-dimethylbenzimidazole. The second, entitled iodotyrosine deiodinase (IYD), catalyzes reductive dehalogenation of halotyrosines. In contrast to NR, BluB and IYD do not utilize NAD(P)H directly but rather require a separate reductase to generate their FMN\textsubscript{hq} in vivo. 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 NS position of the bound FMN. NR provides an amide NH for interacting with the FMN NS, whereas IYD and BluB provide a side-chain OH from Thr or Ser. Due to the importance of the NS position in the redox chemistry of FMN, 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.

IYD was first discovered in humans while the biochemical origins of thyroid disease were being investigated. This enzyme has since been identified in numerous metazoa and certain bacteria but not plants, fungi, or protozoa. Native IYD from bacteria lack a membrane anchor common to the mammalian enzyme, and heterologous expression of the gene from Halicolenobacter hydrossis has produced the most robust deiodinase (hhIYD) to date. This represents a particularly appealing target for environmental engineering since H. hydrossis can be found in sewage treatment plants. Wild-type (wt) hhIYD has been expressed again as a control for the studies described below. The rate constants for deiodination of...
dioiodotyrosine (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 FMNₘₙₚ. This same fluorescence assay has now confirmed that nitrotyrosine (O₂N-Tyr) strongly associates with wt hhIYD, although its reduced to its FMNₙₗₚ form (blue line) by stoichiometric addition of restored almost completely by addition of 10⁻μM dithionite. Thus, only limited reduction of instead generated a signal derived primarily from the neutral oxidation of the FMNₙₗₚ-containing wt hhIYD (18⁻μM FMNₙₘₚ). This same strategy had already been applied successfully when mouse IYD.¹⁷ Stoichiometric addition of dithionite to wt hhIYD under anaerobic conditions reduced its FMNₙₘₚ to FMNₙₗₚ (Figure 1A, eq 1).¹⁹ Thus, only limited reduction of with O₂N-Tyr did not restore the full FMNₙₘₚ spectrum and instead generated a signal derived primarily from the neutral FMNₙₘₚ (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 FMNₙₘₚ had been detected with mouse and human IYD during turnover of Cl-, Br-, and I-YD. In contrast, the inert analogue F-Tyr dramatically stabilized the 1⁻e⁻-reduced FMNₙₘₚ intermediate during redox titration of human IYD. The bacterial enzyme wt hhIYD is now shown to act similarly and readily stabilizes its bound FMNₙₘₚ in the presence of F-Tyr during redox titration with xanthine and xanthine oxidase (Figure 2A).

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

| Protein            | kₐ (min⁻¹) | Kₐ (μM) | kₐ/Kₐ (μM⁻¹·min⁻¹) | K₉ (μM) | K₀ (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|

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 FMNₙₛ. This same strategy had already been applied successfully when first exploring the dechlorinase and debrominase activity of mouse IYD. Stoichiometric addition of dithionite to wt hhIYD under anaerobic conditions reduced its FMNₙₘₚ to FMNₙₙₘₚ as evident from the dramatic loss of absorbance at 450 nm (Figure 1A). Subsequently, the FMNₙₘₚ spectrum was 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 FMNₙₚₘₚ-containing wt hhIYD (18⁻μM). In contrast, treatment of the reduced FMNₙₘₚₘₚ form of wt hhIYD with O₂N-Tyr did not restore the full FMNₙₘₚ spectrum and instead generated a signal derived primarily from the neutral FMNₙₙₘₚ (Figure 1A, eq 1). 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.

The lack of full transfer of electrons from FMNₙₙₘₚ 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 FMNₙₘₚ could be preventing its full discharge of electrons to O₂N-Tyr. Consequently, destabilization of FMNₙₘₚ had the potential to unmask an efficient NR activity of IYD. Such destabilization is already a noted feature of NRs, 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 FMNₙₘₚ intermediate (Figure 2A). 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. However, this residue does contribute to the catalytic efficiency of dehalogenation since the kₐ/Kₐ 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.

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<sub>red</sub> 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<sub>ox</sub> (<10%) as well. This single mutation alone is consequently sufficient to limit formation of the FMN<sub>ox</sub> intermediate and allow full discharge of the reducing equivalents of FMN<sub>red</sub> to the nitro compound. The lack of FMN<sub>red</sub> stabilization by the T173A mutant was similarly evident during its redox titration in the presence of F-Tyr. No FMN<sub>red</sub> 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<sub>red</sub> 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. Most typically, the nitroso intermediate rapidly consumes an additional 2e⁻ from NR to form the corresponding hydroxylamine derivative. Less common but highly desired is the ability of NR to promote full reduction of a nitro compound to its amine derivative.

As a complement to the initial spectrophotometric analysis used to monitor FMN<sub>red</sub> oxidation in hhIYD, consumption of O2N-Tyr was monitored by reverse-phase HPLC (Figure S3). Reaction of O2N-Tyr with the FMN<sub>red</sub> form of either wt or its T173A mutant hhIYD (black points and line), and free FMNH<sub>2</sub> (green points and line) under anaerobic conditions was monitored over time by reverse-phase 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).

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 30 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).

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. The stoichiometry of O₂N-Tyr reduction to H₂N-Tyr by FMNH<sub>2</sub> was also not consistent with contributions from the enzyme alone. Three equivalents of enzyme-bound FMNH<sub>2</sub> 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. 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 FMNH<sub>2</sub> to FMNH<sub>ox</sub> 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 derivative was further confirmed by mass spectrometry (Figure S4). Low yields of the (H₂N-Tyr)dansyl<sub>2</sub> and (O₂N-Tyr)-dansyl<sub>2</sub> 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).

Figure 3. Reduction of O₂N-Tyr by enzyme-bound and free FMNH<sub>2</sub>. 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 hhIYD (black points and line), and free FMNH<sub>2</sub> (green points and line) under anaerobic conditions was monitored over time by reverse-phase HPLC (see Figure S3).
full consumption of three additions of O$_2$N-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; dapsylation of enzyme products; phenylhydroxylamine reduction by bisulphite (PDF)

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### Notes

The authors declare no competing financial interest.

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