

The minimal structure for iodotyrosine deiodinase function is defined by an outlier protein from thermophilic bacteria *Thermotoga neapolitana*

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Abstract:

The nitroreductase superfamily of enzymes encompasses many flavin mononucleotide (FMN)-dependent catalysts promoting a wide range of reactions. All share a common core consisting of a FMN binding domain, and individual subgroups additionally contain one to three sequence extensions radiating from defined positions within this core to support their unique catalytic properties. To identify the minimum structure required for activity in the iodotyrosine deiodinase subgroup of this superfamily, attention was directed to a representative from the thermophilic organism *Thermotoga neapolitana* (TnIYD). This representative was selected based on its status as an outlier of the subgroup arising from its deficiency in certain standard motifs evident in all homologues from mesophiles. We found that TnIYD lacked a typical N-terminal sequence and one of its two characteristic sequence extensions, neither of which was found to be necessary for activity. We also show TnIYD efficiently promotes dehalogenation of iodo-, bromo- and chlorotyrosine, analogous to related deiodinases (IYDs) from humans and other mesophiles. In addition, 2-iodophenol is a weak substrate for TnIYD as it was for all other IYDs characterized to date. Consistent with enzymes from thermophilic organisms, we observed that TnIYD adopts a compact fold and low surface area compared to IYDs from mesophilic organisms. The insights gained from our investigations on TnIYD demonstrate the advantages of focusing on sequences that diverge from conventional standards to uncover the minimum essentials for activity. We conclude that TnIYD now represents a superior starting structure for future efforts to engineer a stable dehalogenase targeting halophenols of environmental concern.

Keywords: flavoprotein, dehalogenase, halotyrosine, reductase, superfamily, nitroreductase

Introduction

The rapid accumulation of genomic information has far outstripped our ability to anticipate the function of the proteins encoded. Predicting structure is a bit less daunting if homologous sequences are available but predicting function is typically very difficult without experimental characterization of many related sequences. The process of selecting the most appropriate representatives to examine has been significantly advanced by the introduction of sequence similarity networks (SSN) that parse large number of sequences within a structural superfamily and identify clusters of related members.(1,2) Even entire metabolic pathways can be discovered by this approach.(3) Such analysis of the nitroreductase superfamily (NTR) encompassing over 24,000 sequences successfully identified numerous clusters of uncharacterized sequences among those with previously determined activity.(4) The predominant features of each subgroup may guide identification of related catalysts but do not necessarily define the essential requirements for sustaining a particular function. As described below, distinct representatives that are remote from the norm best define the minimal features necessary to support reductive dehalogenation by a subgroup within the NTR superfamily. Such information is critical for refining gene annotations and selecting the most expedient parent sequence for engineering new enzyme specificities.

The NTR superfamily extends well beyond nitroreductases and includes the dehalogenase above as well as quinone and quinoline reductases, flavin destructases, and various dehydrogenases. As described by the SSN analysis, a central hub of representatives contain only minimal sequences to form α_2 -homodimers that generate equivalent binding sites for two FMNs within the interface of the subunits (Fig. 1).(4) Radiating away from this hub are more than 14 distinct subgroups that support various activities by the added presence of one, two or three extensions labeled previously as E1, E2 and E3 that emanate from distinct positions within the central homodimer (Fig. 1).(4) For example, the subgroup associated with the well-characterized nitroreductase NfsB contains only an E2 extension whereas the subgroup of flavin destructases associated with BluB contains E1, E2 and E3 extensions. The vast majority of sequences ascribed to iodotyrosine deiodinase

(IYD) contain E1 and E2. These extensions have been postulated to control substrate recognition since they are typically distal to FMN.(4) The central core $\alpha+\beta$ fold is then likely responsible for controlling the redox chemistry of the FMN required for the various mechanisms of substrate transformation.(4) IYD provides an interesting exception since its substrate halotyrosine establishes many of the polar contacts to FMN directly.(5,6)

[insert Figure 1]

Structural characterization of IYDs indicates that E1 and E2 are relatively dynamic.(5-9) E1 appears disordered in the absence of substrate and forms a helix-linker-helix upon substrate coordination. This fold provides an active site lid that sequesters substrate from solvent and activates single electron transfer pathways of FMN as necessary for substrate dehalogenation.(6,7,10) Active site ligands that are unable to stabilize the lid cannot induce dehalogenation and instead allow for an innate nitroreduction by a single two electron transfer.(10) The role of E2 is less clear but is present in mammalian and bacterial IYDs alike.(8) The structure of IYD implies a possible supporting role for E2 based on its contacts to, and presumably stabilization of, the lid structure of E1. Together, E1 and E2 were expected to control the specificity of substrates. IYD was first discovered in humans based on its ability to salvage iodide from iodotyrosine generated in the thyroid although this enzyme and its homologues also promote debromination and dechlorination (Scheme 1).(11-13) The general specificity for halotyrosines is surprisingly consistent throughout metazoa(14) and has prompted efforts to engineer IYDs for accepting halophenols as substrates for the goal of bioremediation.(9) These investigations began using human IYD (HsIYD) as the parent sequence based on the availability of its crystal structure but homologues from thermophilic organisms offer an attractive alternative.(15,16) Additionally, there was hope that IYD from thermophiles might accept a broad array of substrates since the need for a halotyrosine-specific enzyme is not easily rationalized in Bacteria or Archaea. Even the ability of thermophiles to promote dehalogenation could be questioned since none contained E2 sequences in contrast to the homologues from mesophiles. However, studies presented below demonstrate that the minimal structure provided by the thermophilic organism sustains dehalogenation and should offer a robust platform for future engineering of a reductive dehalogenase active in an aerobic environment.

[insert Scheme 1]

Results

Selection of an outlier sequence within the IYD subgroup of the NTR superfamily

The IYD subgroup is evident in all three domains of life but to date most attention has been directed towards the mammalian representatives based on their role in iodide homeostasis.(17-19) The function of this enzyme in other metazoa is not yet known although a link to spermatogenesis has been identified in *Drosophila*(20) and a role in regulating a potassium channel in *Caenorhabditis* has also been suggested.(21) From the SSN illustrated in Fig 2., metazoan sequences cluster together with a notable unique grouping that includes the *Caenorhabditis* sequence. In general, these sequences all contain significant N-terminal extensions beyond the common catalytic core and often include a single transmembrane anchor. A large number of representatives also derive from mesophilic Bacteria. These contain only short N-terminal extensions and lack an anchor for membrane association. IYDs from bacterial and archaeal thermophiles have no N-terminal sequence beyond the catalytic core and are also truncated by 10 amino acids in E2 and one amino acid in E1 (Figs. 2 and S1). Examples from *Pyrococcus furiosus* and *Thermotoga neapolitana* share less than 40% identity with HsIYD and less than 58% identity between each other. We were encouraged to focus on the *T. neapolitana* variant which represented one of the most distant and relatively unique outlier since preliminary data already suggested that even the *P. furiosus* protein maintained some activity as a deiodinase.(8,22) These putative dehalogenases from thermophilies are far from the clusters populated by the mesophilic and psychrophilic organisms from all three domains of life. The structure and catalytic activity of the protein from *T. neapolitana* (TnIYD) are now reported to define the minimal structural requirements for dehalogenation.

[Insert Figure 2]

Expression and purification of TnIYD

TnIYD was expressed in *Escherichia coli* with a C-terminal (His)₆ tag under conditions equivalent to those used to generate IYD from *Drosophila melanogaster* (DmIYD)(14) and *Haliscomenobacter hydroxsis* (HhIYD).(7) Purification included the addition of FMN to the cell lysate to boost FMN occupancy in IYD.

Typically, the addition yields a bright yellow solution due to excess FMN. However, the mixture containing TnIYD and excess FMN rapidly turned dark green. After separation on Ni-NTA resin, the TnIYD fraction appeared purple and exhibited absorption bands at 350, 450, and 590 nm that are typical for a neutral flavin semiquinone (Figs. 3 and S2).(23) After the column-bound TnIYD was washed extensively with air saturated buffer, the purple color dissipated along with its absorbance at 590 nm while absorbance at 450 nm increased. This response is consistent with the expected oxidation of a flavin semiquinone under aerobic conditions. The resulting TnIYD still retained a greenish tint to the expected yellow color of an oxidized flavoprotein (Fig. S2). This tint likely results from the persistence of a weak and broad absorbance near 590 nm (Fig. 3B). No such absorbance was detected previously after preparing IYD from mesophiles but such an observation has been noted for a few other flavoproteins after their formation of charge-transfer complexes between the flavin and various aromatic ligands.(23)

[insert Figure 3]

Identification of the ligand copurifying with TnIYD and preparation of ligand-free TnIYD.

The greenish-yellow preparation of TnIYD was denatured and precipitated with formic acid to release all non-covalent species. Along with FMN, a relatively hydrophilic compound was observed by reverse-phase (C18) HPLC from its absorbance at 275 nm. Subsequent examination by ultraperformance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) identified this compound to be Tyr (Fig. S3). This discovery was quite surprising since no other IYD had previously expressed any measurable affinity for Tyr despite its obvious similarities to the substrate I-Tyr.(6,12) Aromatic ligands co-purifying with other flavoproteins had been released upon FMN reduction(24) and thus TnIYD bound to Ni-NTA resin was treated with dithionite to release the bound Tyr. Re-oxidation of this protein with air-saturated buffer generated the standard yellow flavoprotein without Tyr (Figs. S2B and S3). The various states of TnIYD were also recapitulated individually. First, the purple form was generated by reduction of TnIYD in the presence of Tyr, NADH and an *E. coli* flavin reductase Fre (Fig. S4). Second, the greenish-yellow form with a weak absorbance around 590 nm was formed after adding 1.7 eq. of Tyr to an otherwise Tyr-free preparation of TnIYD containing FMN in its resting, oxidized form (Fig. S5).

Binding and catalysis of TnIYD

Ligand binding to IYD is routinely monitored by the characteristic quenching of fluorescence emitted by the active site FMN in its oxidized form.(6,12) The high affinity of Tyr for TnIYD was verified by this method and the K_d was determined to be $0.45 \pm 0.07 \mu\text{M}$ at 25 °C. The K_d for I-Tyr was even smaller with a value of $0.05 \pm 0.02 \mu\text{M}$. This high affinity is similar to that of the mammalian HsIYD and more than 160-fold greater than that of HhIYD from a mesophilic bacteria. Consistent with all other IYDs characterized to date, TnIYD binds 2-iodophenol (2-IP, $K_d = 31 \pm 1 \mu\text{M}$) significantly weaker than its amino acid derivative, I-Tyr. This suggests that even this thermophilic bacteria maintains a preference for halotyrosines rather than simple halophenols. Such specificity is even more apparent in the K_m values for I-Tyr and 2-IP that differ by 19,000-fold (25 °C, Table 1). Surprisingly, 2-IP is dehalogenated by TnIYD with a k_{cat} value that differs by only 2-fold relative to the k_{cat} value of I-Tyr but k_{cat}/K_m values still indicate a great preference for the halogenated amino acid. Overall, the kinetic constants are best used only for qualitative comparisons since the very low K_m value of I-Tyr and the enzyme's sensitivity to substrate inhibition limit the reliability of these values.

[Insert Table 1]

Enzyme turnover was also measured at an elevated temperature to approach more natural conditions for the thermophile *T. neapolitana*. As expected, TnIYD (T_m 88 °C) maintained structural integrity at much higher temperatures than HsIYD (T_m 45 °C) as observed by CD (Fig. S12). At 60 °C, the k_{cat} and K_m for I-Tyr increased by more than 20-fold relative to the values at 25 °C (Table 1). The large error associated with these values is again due to in part by the limited range of substrate concentrations available before substrate inhibition dominates (Fig. S6).(17) Such inhibition was not observed during 2-IP turnover and instead high concentrations of this substrate were necessary to characterize catalysis. The k_{cat} for 2-IP increased by less than 8-fold after the temperature was raised from 25 °C to 60 °C. The milder effect of temperature on the k_{cat} for 2-IP versus that for I-Tyr may reflect the difficulty of 2-IP to induce a productive orientation of active site side chains but this difference may alternatively result from only the large uncertainty of the values. The k_{cat}/K_m for 2-IP remained more than 10,000-fold smaller than that for I-Tyr at both temperatures. Similar to

IYD from mesophiles (HsIYD and DmIYD), TnIYD also promotes debromination and dechlorination of the suitable halotyrosines. Again, the large uncertainty in the kinetic constants for these substrates limits possible comparisons.

Structural characterization of TnIYD

Crystals of TnIYD bound to Tyr and I-Tyr alternatively were grown by vapor-diffusion and diffracted X-rays to 1.8 Å and 1.6 Å, respectively. The crystal structure of TnIYD bound to Tyr was phased by molecular replacement using a ligand free model generated from the structure of HhIYD bound to I-Tyr (PDB 5KO8).(7) Initial electron-density maps were improved by prime-and-switch density modification(25) followed by iterative rounds of model-building and refinement. Bound FMN and Tyr in each active site along with the entire TnIYD dimer except for the first two residues were visualized in these maps. The final model was refined to an R_{factor} of 15.8% and R_{free} of 20.1% (Table 2). The structure of the I-Tyr•TnIYD complex was determined by difference Fourier using a ligand free model of TnIYD based on the the Tyr-bound structure of TnIYD. The resulting difference maps had clear density corresponding to I-Tyr and the final occupancy of I-Tyr refined to 0.8 (Fig. S13). Overall, this structure refined to an R_{factor} of 17.0% and R_{free} of 20.3% (Table 2). Both crystals had the same unit cell in which the asymmetric unit contained a single copy of the native α_2 -homodimer. The two active sites within each dimer were essentially identical, thus only one representative of each is discussed below.

TnIYD has many structural features common to HhIYD and HsIYD despite sharing less than 40% sequence identity.(6,7) The backbone of TnIYD forms the same signature α + β -fold of the NTR superfamily (Fig. 4) and superposes with RMSD values of 1.5 and 1.4 Å to analogous structures of HsIYD (269 C α atoms) and HhIYD (274 C α atoms), respectively.(6,7) As typical for the IYD subgroup, the active site lid (E1) of TnIYD coordinates directly to I-Tyr but unlike the vast majority of representatives in this subgroup, TnIYD lacks the neighboring E2 domain that interacts with E1 (Figs. 2A, 4 and S1). No other sequence in TnIYD replaces the structural role of the E2 domains present in IYD from mesophiles. This result suggests that the IYD subgroup within the NTR superfamily only requires an E1 sequence to form an active site lid and the core FMN domain to achieve catalytic activity. In addition, the E1 domain of TnIYD is truncated by one amino

acid relative to its mesophilic counterparts without a change in catalytic function or substrate specificity (Figs. 2 and S1). Similarly, the lack of an N-terminal extension in TnIYD relative to other IYDs (Fig. 2) does not adversely affect its catalytic activity nor the thermal stability of its α_2 -homodimer (Fig. S12). Thus, the extended N-terminal region of HsIYD that interacts extensively with its partner subunit is not required for stabilization of the native dimer (see helix of HsIYD highlighted in dark grey, Fig. 4).

[Insert Table 2]

[insert Figure 4]

The E1-based active site lid of TnIYD shares the same motif of two α -helices and a short intervening linker that is observed in IYD from mesophiles. Nearly all interactions between the enzyme, FMN and I-Tyr identified in prior structures are also present in the structure of TnIYD (Fig. 5A). These include polar contacts between the zwitterion of I-Tyr and both the isoalloxazine ring of FMN and the near-universal triad of Glu, Tyr and Lys within E1 (Figs S1 and S7). Similarly, the phenol oxygen of I-Tyr interacts with the 2'-hydroxyl of FMN and a backbone amide proton. In IYD from mesophiles, this amide proton derives from an Ala residue and the presence of this Ala was initially thought to be sufficiently conserved to help parse sequences for the IYD subgroup (Fig. S7).(26) However, variations have since been observed particularly among IYDs from thermophiles. Only 27% of this latter group contains Ala at this position. Replacement of Ala with Met is most typical (62.5%) in IYD from bacterial and archaeal thermophiles including TnIYD (Fig. 5A) and its homologue in *P. furiosus*. Less infrequent substitutions at this site include Ser, Lys and Leu. In no case yet described, does the side chain at this position play a significant role in substrate recognition or structural organization of the active site (see below). Accordingly, the outlier status of TnIYD now demonstrates that neither the E2 domain nor the side chain of the hydrogen bonding donor amide is fundamental to catalysis and substrate specificity.

[insert Figure 5]

The structure of TnIYD with Tyr bound at its active site is nearly identical to that with I-Tyr bound (RMSD of 0.09 Å over 307 C α)(Figs. 5B and S13). Remarkably, all polar contacts between Tyr and TnIYD are equivalent to those between I-Tyr and TnIYD as well as those detected in structures of HsIYD and HhIYD

bound with I-Tyr.(6,7) This observation was not anticipated since the affinity of Tyr to IYD from mesophiles has been too weak to measure whereas binding of Tyr to TnIYD is highly favorable. Still, no structural changes are evident to account for the difference in affinity as only one minor perturbation of a proximal Tyr (Y112, see below) can be detected after Tyr association with TnIYD (Fig. S8). Note too that the complex of Tyr and TnIYD containing the oxidized FMN may represent a final intermediate of a catalytic cycle. Only the halide is absent from this complex and the order of its departure is not yet known.

Molecular basis for the unusually tight binding of Tyr to TnIYD

A small shift in Y112 from a position labeled “out” to one labeled “in” is evident when comparing the structures of TnIYD bound with I-Tyr and Tyr, respectively (Fig. S8). While this minor conformational change is unlikely to be the sole basis for the high affinity of TnIYD for Tyr, its contribution was examined with a Y112A mutation. This variant bound both I-Tyr and Tyr with nearly the same affinities as the native TnIYD and thus the position of the aromatic side chain was not sufficient to stabilize Tyr association (Table 3).

[Insert Table 3]

The role of M41 was next examined by a M41A mutation. As noted above, Ala is the predominant residue at this position for IYDs from mesophiles. Additionally, the side chain of Met41 adopts multiple conformations in the crystal structure of the TnIYD·Tyr complex (Fig. 5B). The M41A substitution did weaken the enzyme’s affinity for Tyr by 3.6-fold but its effect on I-Tyr was slightly greater (5-fold, Table 3). None of these individual substitutions in TnIYD could recreate the high selectivity of other IYDs for I-Tyr relative to Tyr and a variant with substitutions of M41A and Y112A in TnIYD also failed at limiting Tyr binding. The affinity for Tyr by this variant decreased by only 2-fold while the affinity for I-Tyr increased by 50%. Lys is a natural variant of M41 in IYD and offers the contrast of a charged side chain. Substitution of this residue in a M41K variant of TnIYD decreased affinity for I-Tyr and Tyr by 22-fold and 16-fold, respectively (Table 3). An alternative M41F variant was examined to test if a nonpolar aromatic side chain could selectively impede the binding of Tyr. Indeed, this variant affected ligand binding but in the opposite manner than originally anticipated. Substitution of Phe for the native Met, enhanced binding of Tyr by 5-fold and concurrently decreased binding of I-Tyr by 10-fold resulting in a protein with an inverted selectivity that

now favored Tyr over I-Tyr by 5-fold. Thus, none of these individual changes could be responsible for loss of the high selectivity of I-Tyr over Tyr for IYDs from mesophilic organisms.

The ability of TnIYD to bind Tyr could instead derive from a decrease in surrounding polarity relative to HsIYD. Besides Y112, Tyr and I-Tyr are surrounded by W82 and F88 in the active site of TnIYD (Fig. S9). Y212 in HsIYD acts as an equivalent to Y112 in TnIYD but the two remaining aromatic residues in TnIYD are replaced with D172 and T178 in HsIYD. To examine the role of these aromatic amino acids in TnIYD, a triple mutant was prepared in which all three aromatic amino acids were substituted with Ala (W82A/F88A/Y112A). This modification noticeably weakened binding of I-Tyr by 18-fold but also re-established selectivity for this substrate by a much more substantial destabilization of Tyr binding of more than 2600-fold (Table 3). Thus, the aromatic residues in the TnIYD active site provide little specificity for I-Tyr and instead create a nonpolar environment for nonspecific association of an aromatic group.

Discussion

The NTR superfamily encompasses a FMN-binding core that has accumulated one or more extensions at common sites to create a number of subgroups promoting a range of reactions.(4) The full diversity of catalysis remains to be described but knowledge to date has already allowed application of this superfamily to numerous topics such as synthetic chemistry,(27,28) prodrug therapy,(29) biomedical imaging,(30) cell biology(31) and bioremediation.(9,32) Curation of this superfamily through a SSN provides a powerful guide to discover the structural and evolutionary basis for the FMN-dependent processes.(4) In particular, interest in IYD derives from its unique ability to promote reductive dehalogenation under aerobic conditions in the absence of metals and chalcogens.(8,33) This dehalogenase also has the potential to serve a variety of applications but its narrow range of substrates currently restricts its utility. To guide future manipulation of this system, we examined a representative on the periphery of the IYD subgroup to establish the minimal requirements for catalysis. A variety of predominant features of this subgroup have now been deemed non-essential by this approach.

Substrate selection and ligand affinity

How Bacteria and Archaea may be exposed to halotyrosines has not yet been studied but the presence of halophenol derivatives in the environment are widespread.(34-36) Still, TnIYD demonstrates a stringent specificity for halotyrosines that is consistent with its three conserved amino acids (E68, Y72, K92) within the E1 domain that coordinate the zwitterion of these amino acids (Fig. 5). For comparison, 2-IP is dehalogenated with an efficiency (k_{cat}/K_m) of less than 0.01% of that for iodotyrosine at 25 °C (Table 1). This difference is likely due to the necessity of the substrate's zwitterion to organize the active site for efficient turnover as manifest in the K_m value that extends over four orders of magnitude from 2-IP to I-Tyr.

TnIYD defied expectations by its ability to bind Tyr with high affinity. No other IYD has yet to demonstrate a measurable affinity for tyrosine.(6,12) Previously, high affinity of an active site ligand was predicated by substitution on the phenolic ring with a halogen or other substituent that enhanced the acidity of the phenolic proton.(8) These observations were considered indicative of a preference for IYD to bind the phenolate form of its ligands. Only minute quantities of the ionized form of Tyr are present under standard conditions of pH 7.4. The affinity of TnIYD for the neutral form of Tyr is likely based on the introduction of aromatic side chains (W82, F88) that enhance the nonpolar properties of its binding site relative to that of IYDs examined from mesophiles (Fig. S9). Although a phenolic hydrogen is modeled in the crystal structure of TnIYD, the exact protonation state of bound Tyr remains to be determined. The distances between its phenolic oxygen and the hydrogen bond donor from the polypeptide backbone are all between 2.7 - 3.0 Å in the crystal structures of I-Tyr bound to HsIYD, HhIYD, TnIYD and IYD from *Mus musculus* (MmIYD) (Fig.5).(5-7) The phenolic oxygen of Tyr is also within 2.6 Å of the hydrogen bond donating 2'-hydroxy of FMN, a distance identical to that observed in all of the structures noted above for the phenolic oxygen of I-Tyr.

A corollary to the high affinity of TnIYD for Tyr is readily observed after heterologous expression in *E. coli*. As isolated, TnIYD appears purple rather than the usual yellow of a flavoprotein. The origins of this derive from the ability of TnIYD to scavenge endogenous Tyr from the host bacteria. Binding of Tyr stabilizes closure of the active site lid formed by the E1 sequence and this in turn should stabilize the one electron reduced neutral semiquinone form of FMN that is apparent in the UV-vis spectrum (Fig. 3). Such stabilization was observed previously after fluorotyrosine (F-Tyr), an inert substrate analogue, bound to HsIYD.(6,10) The

surprising longevity of the semiquinone form of TnIYD under aerobic conditions suggests that active site lid closure is highly efficient at protecting the cofactor from solvent and its accompanying dissolved molecular oxygen. Otherwise, reaction between the semiquinone and molecular oxygen would be extremely rapid.(37)

Minimal structure required for reductive dehalogenation

E2 sequences are nearly omnipresent in the IYD subgroup of the NTR superfamily.(4) Based on this observation alone, such a domain would seem essential (Fig. 2). In HsIYD, E2 forms a loop that protrudes from the otherwise rather globular profile of the homodimer (Fig. 4). A catalytic function for E2 was not expected since this loop extends away from the active site and lacks residues that are directly involved in binding substrate or FMN. A structural role is more likely due to the observed π - π stacking at a distance of 3.3 Å between a highly conserved Tyr (Y211) proximal to E2 and an equally conserved Trp (W169) within the active site lid formed by the E1 sequence (Fig. 6A). This interaction has the potential to stabilize closure of the lid and facilitate the disorder to order transition of E1 upon substrate binding. Characterization of TnIYD has now demonstrated that such an interaction is not necessary. Moreover, the entire E2 is dispensable for substrate recognition and turnover. Again, no compensatory changes in the structure of TnIYD replace the interactions lost by the absence of an E2 sequence. The closest approach between the E1 lid and remnants of E2 in TnIYD are between a Pro (P111) and Leu (L79) at a distance of 4.7 Å (Fig. 6B). Not surprisingly, neither equivalents of Y211 or W169 are conserved in TnIYD.

Unlike the domains above, E1 is essential for all eukaryotic, bacterial and archaeal IYDs regardless of the preferred growth conditions. This region is dynamic in the absence of substrate but adopts a helix-linker-helix in the presence of substrate to form an active site lid.(5-7) This organization also brings a Thr side chain proximal to the N5 position of FMN for stabilizing an intermediate FMN semiquinone and promoting a series of single-electron transfers necessary for reductive dehalogenation.(6,7,10) Likewise, dehalogenation is very inefficient for substrates such as 2-IP that are unable to organize E1 into a lid structure (Table 1). Without the conformational changes associated with lid closure, the FMN is only capable of hydride transfer and will instead promote nitroreduction in analogy to other subgroups within the NTR family.(7,10) Most important within the E1 sequence are the universally conserved Lys and Glu (E68, K92 in TnIYD, Fig. 5) and the highly conserved Tyr

(Y72) that form polar bonds to the zwitterion of halotyrosine. The significance of these interactions had previously been demonstrated by mutagenesis.(14,38) In particular, replacement of this Glu to Gln suppresses dehalogenation severely for both MmIYD and DmIYD.

Thermophilic adaptation

Many factors contribute to thermal stability of proteins. In comparison to mesophilic counterparts, proteins of thermophiles are often enriched in hydrophobic and β -branched amino acids as well as Pro and depleted in uncharged polar residues.(39) Such trends are not apparent when comparing TnIYD to HsIYD and HhIYD other than the gain of hydrophobic residues (W82, F88) in the active site of TnIYD and even these substitutions are not universal in all homologues from thermophiles. An increase in the number of salt bridges is another common strategy for achieving thermal stability(39-41) but a similar number of salt bridges is observed within HsIYD and TnIYD. Instead, the thermal stability of TnIYD likely derives from its minimal sequence that is shared by homologues from other thermophilic Bacteria as well as thermophilic Archaea and distinct from those of mesophiles from all three domains of life.(4) Unique to IYD from thermophiles are (i) the lack of an N-terminal sequence extending from its catalytic core, (ii) loss of one amino acid from the E1 region and (iii) loss of 10 amino acids that form the E2 region (Fig. 2). The absence of these residues diminishes the overall surface area of the homodimer for TnIYD ($16230 \text{ \AA}^2$) relative to HsIYD ($18550 \text{ \AA}^2$) by 14% (Figs. 7 and S10). Furthermore, the absence of E2 removes a relatively dynamic region that is likely most sensitive to thermal denaturation. In the crystal structure of HsIYD, the average B-factor of E2 ($64 \text{ \AA}^2$) is 40% larger than that of its core and in HhIYD the corresponding average B-factor for E2 ($80 \text{ \AA}^2$) is almost 80% larger than that of its core (Fig. S11). Thermophilic adaptation within the IYD subgroup of the NTR superfamily therefore provides instruction for generating stable IYD derivatives and illustrates the minimal structure required for catalytic activity despite their status as outliers from the vast majority of IYD homologues identified to date.

Conclusion

Analyzing large superfamilies of proteins to create SSN is a powerful tool for discovering structurally distinct subgroups that can reveal key determinants for diagnosing function.(1,2) Recent evaluation of the NTR

superfamily revealed radial development of function emanating from a central hub of proteins with a minimal FMN-binding domain.(4) Each functional subgroup was dominated by a specific combination of extensions (E1, E2 and/or E3) that may help to determine catalytic specificity. At least for the IYD subgroup, however, the structural requirements for activity are not defined by the prevalence of particular features. Instead, these have been identified from the exceptional sequences of a small set of IYDs from thermophiles. Neither an extended N-terminal extension, a loop formed by E2 nor an Ala at the site of substrate-backbone hydrogen bonding are unnecessary for dehalogenating halotyrosines. The minimal presence of an E1 sequence forming an active site lid and the core FMN-binding domain are instead sufficient to support catalysis. Prior analysis of IYD from *P. furiosus* and more detailed studies on TnIYD now illustrate the conservation of catalytic function and substrate specificity for IYD ranging from mesophiles to thermophiles in all three domains of life. This far exceeded the original expectation for IYD's distribution based on its initial association with iodide homeostasis in chordata.(4,26)

Experimental procedures

Plasmids

The original plasmid (pET28) encoding TnIYD (NCBI accession: ACM22745.1) was a generous gift from Dr. Janine Copp (University of British Columbia, Vancouver). The TnIYD gene was subcloned into a pET24a vector (Novagen) via restriction sites NdeI and XhoI that were introduced by PCR (see Table S1 for primers). A C-terminal (His)₆ tag was then added to the TnIYD sequence by mutagenesis using standard protocols.(42,43) The resulting construct was used as the template for further mutagenesis to generate the variants listed on Table S1. The gene encoding flavin reductase (Fre) was amplified from the *E. coli* genome (Genehogs, Invitrogen) with a C-terminal (His)₆ tag and cloned into a pET24a vector via overlap extension PCR.(44) All oligonucleotides were synthesized by IDT and Sigma and all PCR reactions utilized Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). The desired genes were all verified by Sanger sequencing (Genewiz).

Protein expression and purification

Plasmids encoding the desired TnIYD gene and its variants were transformed into Rosetta2 chemically competent *E. coli* (Novagen). The culture was then grown in 500 ml LB media at 37 °C with vigorous shaking (220 rpm) before cooling to 16 °C after an OD₆₀₀ of ~ 1 was observed. Isopropyl thio-β-galactoside (IPTG, 25 μM final, Goldbio) was added and the cultures were shaken (220 rpm) for ~15 hr at 16 °C. Cells were harvested by centrifugation (5000×g, 10 min) at 4 °C, flash-frozen in liquid nitrogen and stored at -80 °C. Fre was expressed using the same conditions as TnIYD. Frozen cell pellets containing TnIYD (~ 4 g) were thawed at room temperature and resuspended by vortexing in ~ 25 ml cell lysis buffer (50 mM sodium phosphate pH 8.0 with 500 mM sodium chloride, 10% glycerol, 25 mM imidazole and 0.5 mM tris(2-carboxyethyl)phosphine (TCEP)). All subsequent procedures were performed at 4 °C unless otherwise noted. FMN was added to the suspension to a final concentration of ~ 300 μM and subsequent cell lysis used an EmulsiFlex-C3 homogenizer (Avestin, 3 passes at ~17000 psi). Cell debris was removed by centrifugation at 45000×g for 30 min. The resulting supernatant was loaded onto Ni-NTA resin (5 ml) (Thermo Fisher Scientific, pre-equilibrated with 10 column volumes of lysis buffer) by a bench top Econo pump (Bio-rad) at a flow rate of 1 ml/min. The column was then washed with 16 column volumes of air-saturated washing buffer (50 mM sodium phosphate pH 8.0 with 500 mM sodium chloride, 10% glycerol, 60 mM imidazole and 0.5 mM TCEP) using an ÄKTA protein purification system (GE Healthcare) at 1 ml/min. Tyr-bound TnIYD was eluted with elution buffer (50 mM sodium phosphate pH 8.0 with 500 mM sodium chloride, 10% glycerol, 250 mM imidazole and 0.5 mM TCEP). To isolate Tyr-free TnIYD, the column was alternatively washed with 4 column volumes of cell lysis buffer supplemented with sodium dithionite (2% final, technical grade, Sigma-Aldrich) with an Econo pump at 1 ml/min. The resulting colorless protein was then washed with air saturated washing buffer to reoxidized TnIYD (bright yellow). This species was eluted as described above with elution buffer. Samples were then incubated with ~300 μM FMN on ice for 1 h before an exchange to storage buffer (50 mM sodium phosphate pH 7.4 with 100 mM sodium chloride, 1 mM TCEP and 10% glycerol) using a gravity driven PD-10 desalting column (GE healthcare) following manufacturer's instructions. Purified protein was finally concentrated with a 30 kD molecular weight cut off centrifugal filter unit (Amicon), flash-frozen with liquid nitrogen and stored at -80 °C. Fre was purified by the same method and all enzymes were analyzed by 12% SDS-PAGE with Comassie brilliant

blue staining. Enzyme concentration was determined by UV-vis absorption of enzyme-bound FMN (ϵ_{450} 12500 M⁻¹cm⁻¹).⁽⁴⁵⁾ FMN occupancy was estimated by the ratio of enzyme-bound FMN over the total protein concentration that was calculated from A₂₈₀ (extinction coefficient estimated by the ExPASy ProtParam)⁽⁴⁶⁾ as described previously.⁽⁶⁾

Identification of the low molecular weight ligand that co-purifies with TnIYD expressed in E. coli

The greenish-yellow form of TnIYD (80 μ M) in storage buffer was diluted with 1 equiv. of water and supplemented with 4.4% formic acid. This solution was incubated at room temperature for 5 min before centrifugation at 14000 $\times$ g for 5 min. The supernatant (200 μ l) was fractionated using a Jasco HPLC with two PU-980 Intelligent pumps, a MD-1510 photo-diode array detector and a solvent gradient listed in Table S2. The ligand was collected after chromatographic separation and dried under reduced pressure (Savant Speed Vac, SC100A). The resulting residue was dissolved in water (120 μ l) and analyzed with a Waters Acquity/XevoG2 UHPLC-MS system using an Acquity UPLC[®] HSS T3 column (100 Å, 1.8 μ m, 2.1 mm $\times$ 100 mm) and a binary mobile phase of water (A) and acetonitrile (B) with 0.1% v/v formic acid in each at a flow rate of 0.3 μ l/min. Compound separation was achieved with an initial B from 0 – 5% over 1 min and then continued to 60% B for the next 6 min with a column temperature of 35 °C. Positive electrospray ionization mode was used for detection with a capillary voltage, sampling cone and extraction cone set to 3 kV, 40 V, and 4 V, respectively. The source temperature was 130 °C and desolvation gas temperature and flow rate were 400 °C and 400 l/hr, respectively. All mass spectrometer data were analyzed by Masslynx v4.1 (Waters).

Crystallization and structure determination of TnIYD

Complexes between TnIYD and ligands were formed by incubating approximately 10 mg/ μ l of TnIYD with 2- and 3-fold molar excess of Tyr and I-Tyr respectively in 10 mM Tris pH 7.4 with 100 mM sodium chloride for three hours at 4 °C. Crystals were grown by hanging-drop vapor diffusion after mixing ratios (1:1 and 2:1) of complex to well solution containing 100 mM sodium citrate pH 4.5 with 3 M NaCl. Plates were then incubated at 20 °C. Bright yellow plate-shaped crystals appeared after one day and grew to their maximum size in a week with approximate dimensions of 400 $\times$ 150 $\times$ 40 μ m. Crystals were then transferred directly from the drop into a cryo-stabilization buffer (100 mM citric acid pH 4.5 with ~2 mM of ligand, 3 M sodium chloride and

30% PEG400) and flash frozen in liquid nitrogen. Diffraction data were collected at beamline 12-2 at the Stanford Synchrotron Radiation Lightsource and processed with XDS/Aimless.(47) For the initial structure, phases were determined by iterative rounds of molecular replacement with a search model corresponding to a ligand free, trimmed, polyalanine monomeric model of HhIYD (PDB 5KO8)(7) in Phaser.(48) Initial electron-density maps were improved by prime-and-switch density modification.(25) Subsequent structures were determined by difference Fourier. Models were built by iterative rounds of manual-building in Coot(49) and refinement in Phenix.(50) The occupancy of the ligands was refined manually by setting the occupancy and evaluating the difference Fourier maps. Polder omit maps were calculated in Phenix.

Structural Analysis

Distance measurements were calculated and structure figures were rendered in Pymol.(51) Buried surface area calculations were performed in PDBePISA.(52,53)

Binding affinity of active site ligands

Active site binding is reported as dissociation constants (K_d) with TnIYD and its variants and determined by ligand-dependent quenching of the fluorescence of FMN_{ox} as described previously.(12,54) Briefly, A solution of ligand was titrated with mild stirring into 2 - 4 μ M enzyme in 100 mM potassium phosphate pH 7.4 and 200 mM potassium chloride at 25 ± 1 °C. Fluorescence emission was measured at 516 – 526 nm when excited at 450 nm. The ratio of observed to initial fluorescence (after correcting for dilution) versus ligand concentration was fit to a two-state binding isothermal.

Catalytic dehalogenation

Rates of catalysis were measured by formation of the dehalogenated products Tyr or phenol using reverse-phase HPLC as previously described.(7) Briefly, 50 nM to 2.5 μ M enzyme was incubated with various concentrations of halotyrosine or 2-IP in 220 mM potassium chloride and 110 mM potassium phosphate pH 7.4 (900 μ l) at the assay temperature for 5 min. Sodium dithionite (5%) in 5% sodium bicarbonate (100 μ l) was then added to initiate catalysis. Reaction was quenched by adding a 1:1 mixture (100 μ l) of aq. formic acid (88%) and Cl-Tyr (0.55 μ M final in the assay buffer as an internal standard). The resulting mixture was separated and quantified (A_{271}) by reverse-phase C18 (Agilent, Microsorb-MV 300 C18, 250 $\times$ 4.6 mm) HPLC using solvent

gradients (1 ml/min) summarized in Table S2. Rate versus substrate concentration was fit to the Michaelis-Menten equation (Origin 9.0) (Fig. S6).

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Author contributions

Z.S. performed the cloning, expression and general characterization of TnIYD. Z.S. and B.X. generated and characterized mutants of TnIYD. S.S. measured dehalogenation of 2-IP. Z.S. crystallized the proteins and J.K. solved their structures. S.R. supervised all aspects of the project and all authors discussed the results and contributed to the final presentation.

Data availability

The structures presented in this paper have been deposited in the Protein Data Bank (PDB) with the codes 6PZ0 and 6Q1L.

Conflict of interest

No conflict to report

Funding and additional information

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Supporting information

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Table 1. Steady-state properties of TnIYD.^a

	substrate	k_{cat} (min ⁻¹)	K_M (μ M)	k_{cat}/K_M (μ M ⁻¹ min ⁻¹)
25 °C	I-Tyr	1.6 ± 0.1	0.22 ± 0.07	7.2 ± 2.4
	2-IP	0.84 ± 0.06	$(4.2 \pm 0.7) \times 10^3$	$(2.0 \pm 0.4) \times 10^{-4}$
60 °C	I-Tyr	27 ± 11	3.5 ± 2.5	10 ± 6
	Br-Tyr	5.1 ± 0.3	0.14 ± 0.12	36 ± 31
	Cl-Tyr	2.9 ± 0.6	5 ± 2	0.6 ± 0.3
	2-IP	6.5 ± 0.3	$(6.6 \pm 0.5) \times 10^3$	$(9.9 \pm 0.9) \times 10^{-4}$

^[a]All data and error were obtained by fitting a minimum of two independent measurements to standard Michaelis-Menten kinetics.

Table 2. Data collection and refinement statistics of TnIYD structures.

PDB ID	6PZ0	6Q1L
Protein	TnIYD	TnIYD
Ligand	Tyr	I-Tyr
Data Collection		
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit Cell		
a,b,c (Å)	42.88, 81.82, 103.75	42.86, 81.23, 103.90
$\alpha=\beta=\gamma$ (°)	90	90
Number of unique reflections ^a	34678 (1965)	47126 (2264)
Number of observed reflections ^a	225230 (11584)	211852 (9484)
Resolution (Å) ^a	35.67-1.80 (1.84-1.80)	37.91-1.60 (1.63-1.60)
R _{pim} (%) ^{a,b}	4.3(13.8)	3.9 (38.0)
I/ σ ^a	15.1(5.1)	10.7 (2.1)
Completeness (%) ^a	99.8 (97.7)	96.9 (95.5)
Redundancy ^a	6.5 (5.9)	4.5 (4.2)
Refinement		
R _{cryst} (%) ^{a,c}	15.83 (16.63)	17.04 (22.607)
R _{free} (%) ^{a,c}	20.10 (21.59)	20.26 (25.27)
Ramachandran analysis		
Favored (%)	97.85	98.63
Allowed (%)	2.15	1.37
rmsd bonds	0.007	0.010
rmsd angles	1.051	1.3
Average B-factor for protein (Å ²)	23.55	21.81
Average B-factor for water (Å ²)	31.15	31.30
Average B-factor for ligands (Å ²)	15.21	14.49

^aThe values in parentheses are for the highest resolution shell.

^b $R_{pim} = \sum_{hkl} \sqrt{((1/(n-1)) \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i)}$, where I_i is the intensity of an individual reflection and $\langle I \rangle$ is the mean intensity obtained from multiple observations of symmetry related reflections.

^c R_{cryst} is $\sum \|F_o - F_c\| / \sum F_o$, where F_o is an observed amplitude and F_c a calculated amplitude; R_{free} is the same statistic calculated over a subset of the data that has not been used for refinement.

Table 3. Binding affinity of I-Tyr and Tyr as a function of side chain substitution (25 °C).^[a]

TnlYD	K_d (μ M)	
	I-Tyr	Tyr
wt	0.05 ± 0.02	0.45 ± 0.07
Y112A	0.03 ± 0.02	0.9 ± 0.1
M41A	0.25 ± 0.06	1.6 ± 0.2
M41A/Y112A	0.024 ± 0.002	0.95 ± 0.09
M41K	1.1 ± 0.1	7.4 ± 0.2
M41F	0.5 ± 0.1	0.09 ± 0.06
Y112A/W82A/F88A	0.9 ± 0.1	1200 ± 20

^[a]All data and error were obtained by fitting of two independent measurements.

Figure legends

Scheme 1. Reductive dehalogenation promoted by iodotyrosine deiodinase (IYD)

Figure 1. The minimal scaffold for binding FMN in the NTR superfamily. (A) A two dimensional schematic for one of two polypeptides in the α_2 -homodimer indicates regions of α -helix (α), β -strand (β) and the sites for sequence extensions (E1, E2, E3). (B) A three dimensional representation of a native homodimer in which the individual polypeptides are depicted in grey and wheat. This structure is based on a protein (pdb 3E39) within the central hub as described by Akiva et al. (4). FMN is illustrated as a stick drawing with carbon atoms in orange.

Figure 2. Diversity of the IYD subgroup within the NTR superfamily. (A) Prototypic variations in polypeptide length for a few representative homologues of Human IYD (HsIYD). Amino acid identity with respect to HsIYD is included in parenthesis. (B) Sequence similarity network of 625 putative IYD sequences from the NTR superfamily (e value of 5, edge alignment of 62) (4). Red diamonds, green ovals, light blue rectangles and dark blue ovals represent sequences within archaea, prokaryotes, eukaryotes and unknown origins, respectively.

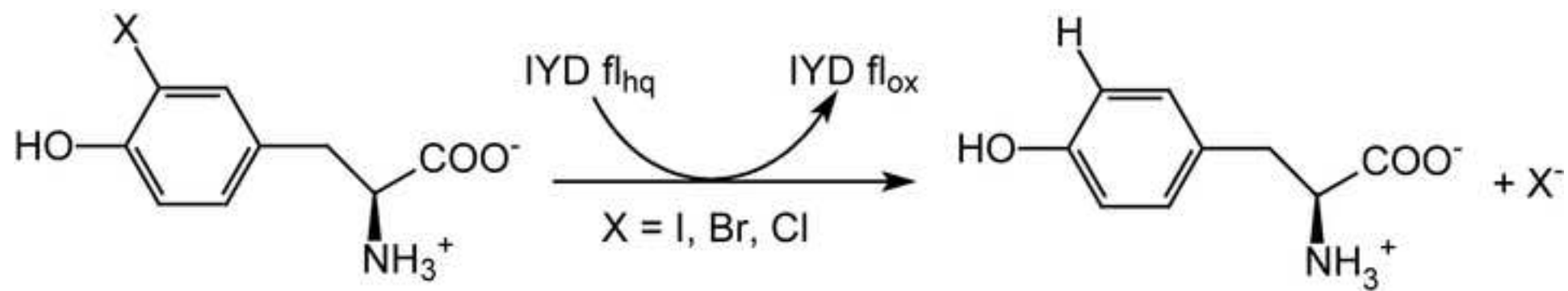
Figure 3. UV-vis spectra of TnIYD isolated after heterologous expression. (A) Purification of TnIYD on Ni-NTA resin produced a purple solution. (B) Extensive washing with air-saturated buffer prior to elution produced a yellow-green solution. The insert expands the scale to illustrate the low absorbance at long wavelengths. Enzymes were diluted in 110 mM potassium phosphate pH 7.4 with 220 mM potassium chloride under ambient conditions prior to recording the absorbance spectra (See Figure S2 for more details).

Figure 4. Comparison of TnIYD•I-Tyr (PDB 6Q1L) and HsIYD•I-Tyr (PDB 4TTC). Polypeptides of the α_2 -dimer are colored in light grey and wheat. FMN and I-Tyr are colored with orange and yellow carbons, respectively. E1 and E2 extensions are colored in blue and red. The extended N-terminus of HsIYD is colored in dark grey.

Figure 5. Comparison of the active sites TnIYD bound alternatively with I-Tyr (PDB 6Q1L) and Tyr (PDB 6PZ0). Cartoon representations of (A) I-Tyr and (B) Tyr bound to TnIYD. Individual polypeptides of the α_2 -dimer are colored grey and wheat. E1 is colored blue. Carbons of FMN are colored orange and carbons of I-Tyr and Tyr are colored yellow. Sulfur of the active site Met41 is also indicated in yellow and was observed in two orientations with bound Tyr. Interactions between ligands and TnIYD (≤ 3.5 Å) are indicated by dashed lines.

Figure 6. Contact between E1 and E2 in (A) HsIYD•ITyr (PDB 4TTC) and (B) TnIYD•ITyr (PDB 6Q1L). Individual polypeptides of the α_2 -dimer are colored grey and wheat. Carbons of FMN are colored orange and carbons of I-Tyr are colored in yellow. E1 and E2 are colored with blue and red, respectively and the shortest distance between these two regions is indicated by a dotted line.

Figure 7. Increasing surface area from a protein of the NTR hub to TnIYD and HsIYD. (A) The core FMN-binding domain is represented by the structure of a protein within the NTR hub (PDB 3E39) (4). (B) TnIYD (PDB 6Q1L) and (C) HsIYD (PDB 4TTC) are represented by their complexes with I-Tyr. The positions of E1, E2 and E3 are noted in blue, red and green as introduced in Fig. 1. FMN is colored with orange carbon atoms. See Fig. S10 for full view of the front and back of these structures.



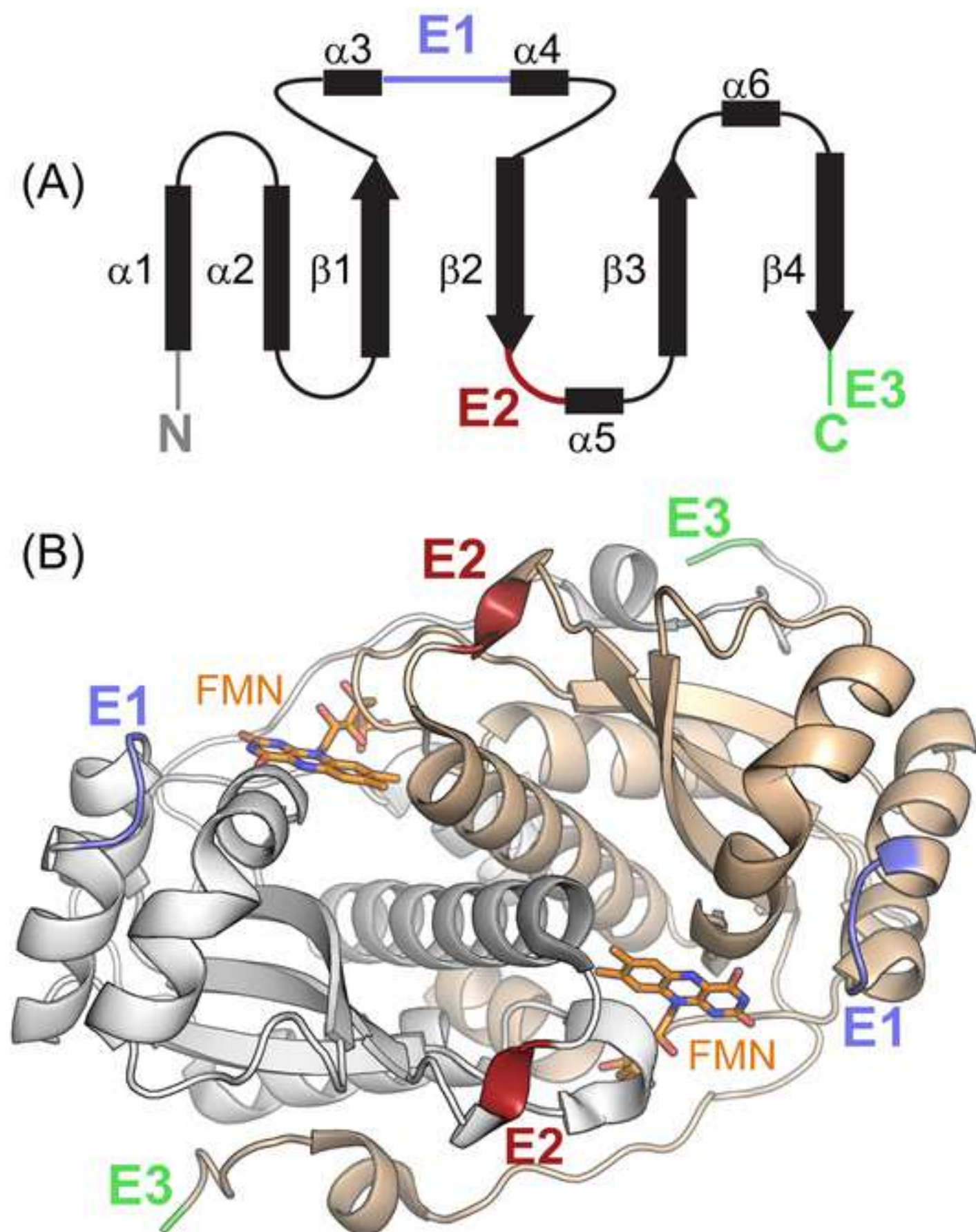
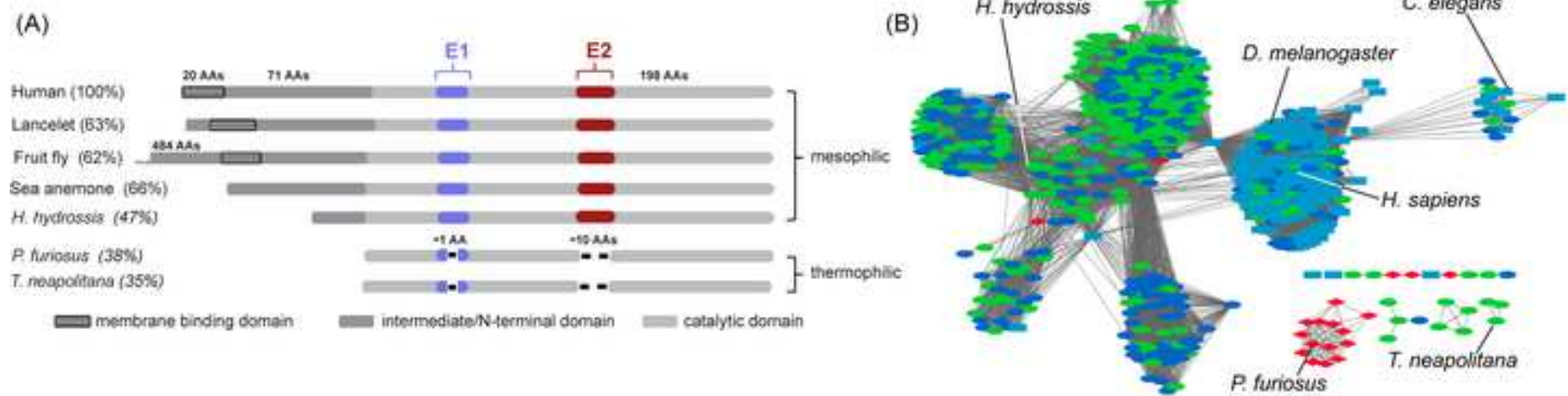


Figure 2



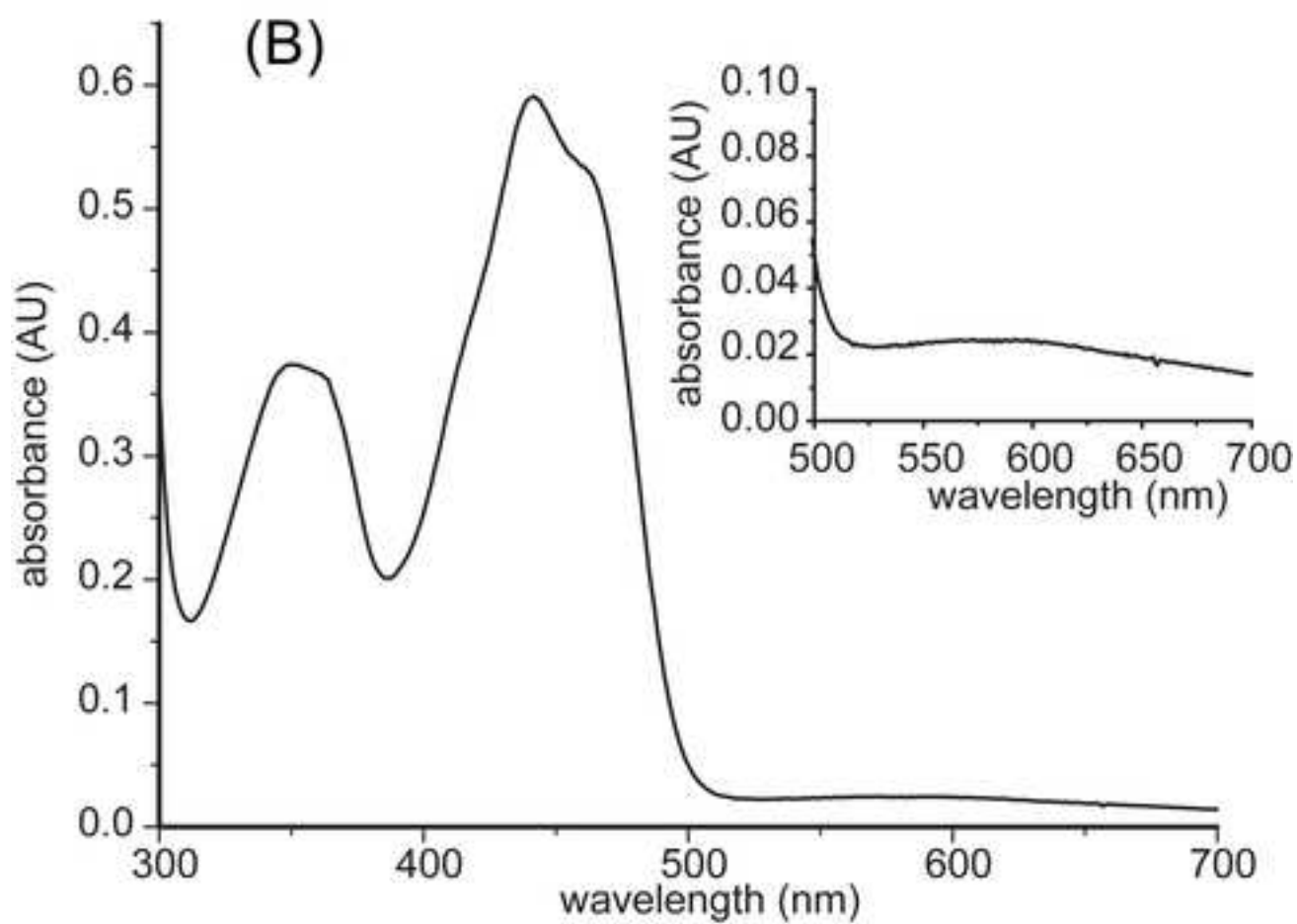
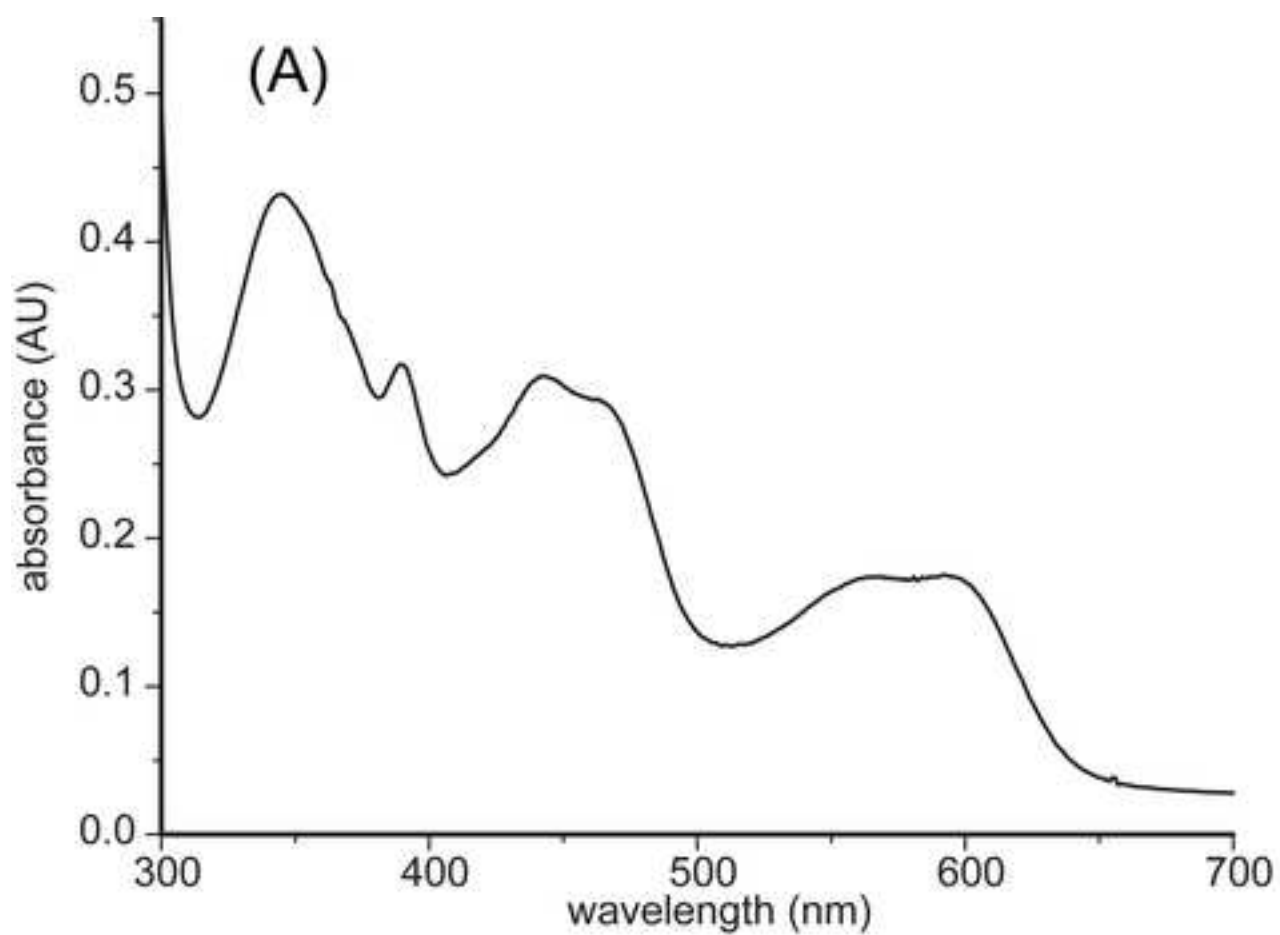
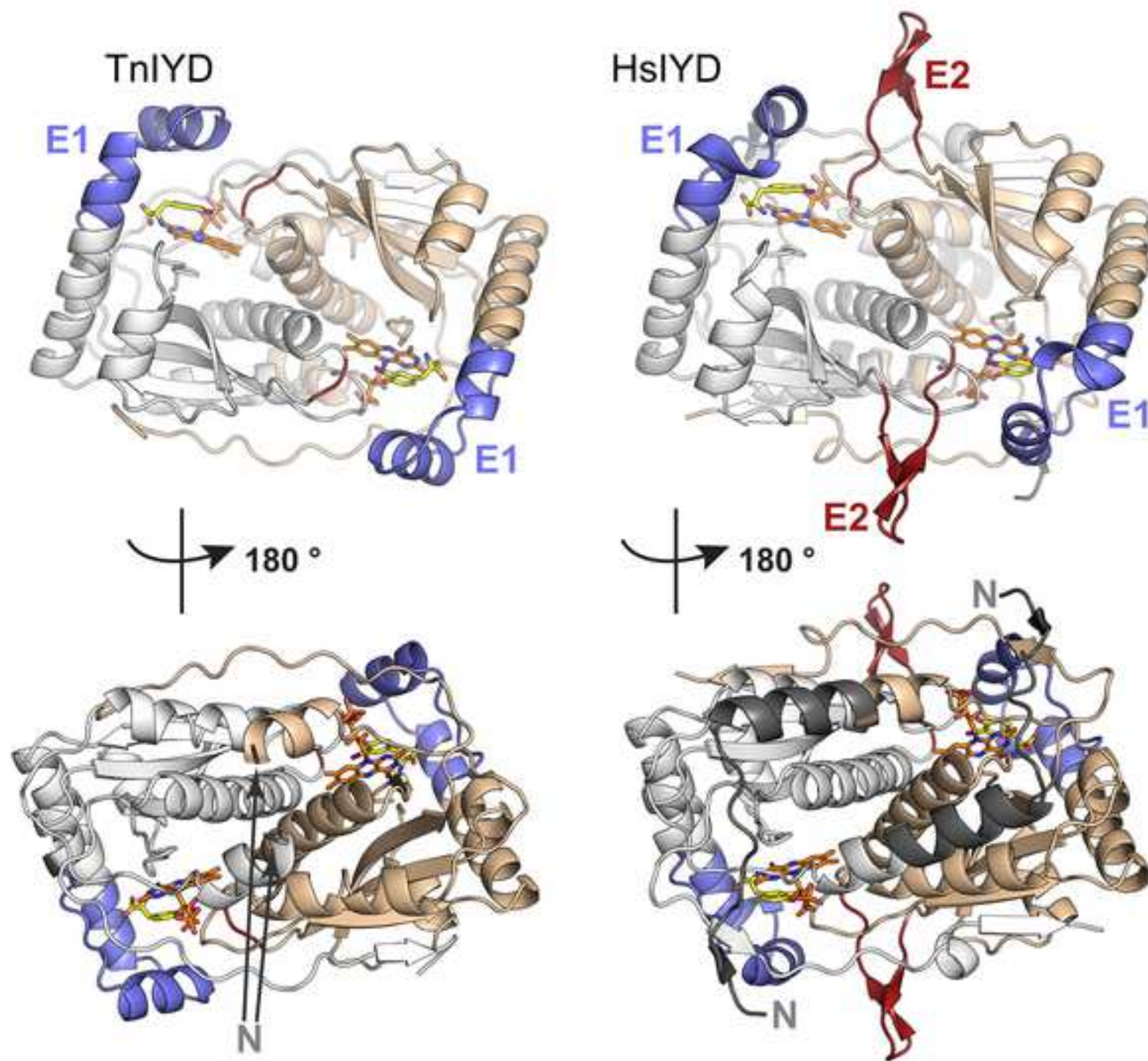
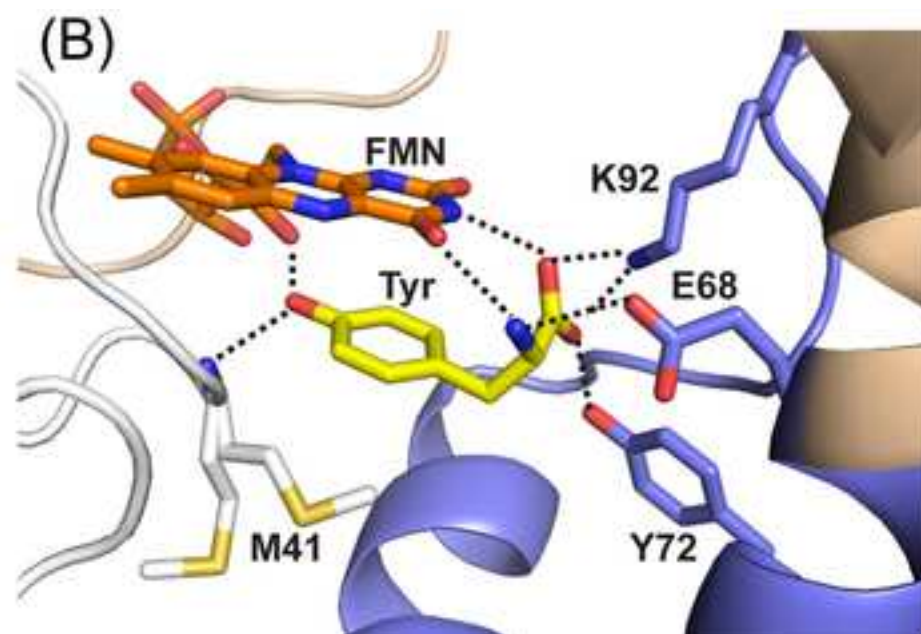
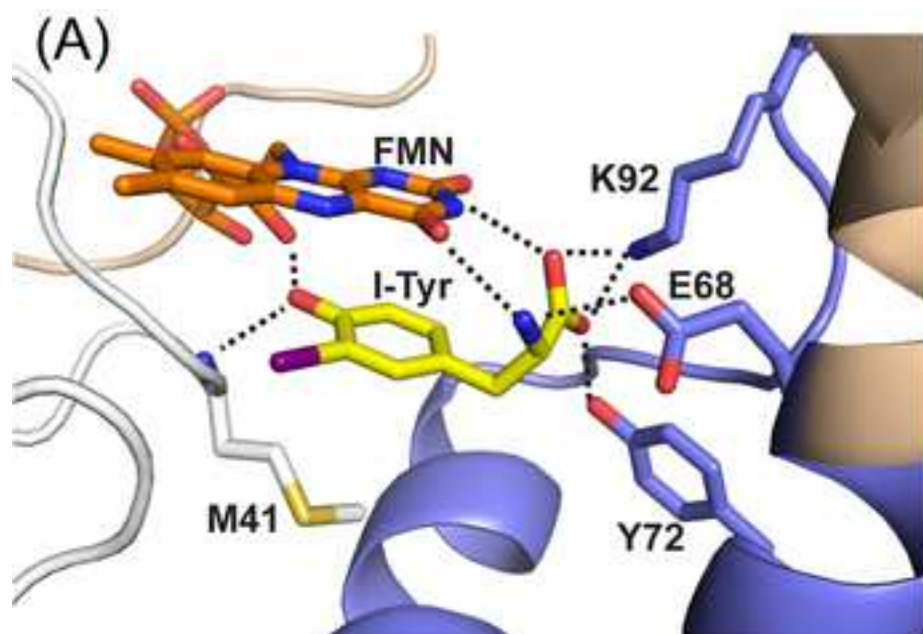
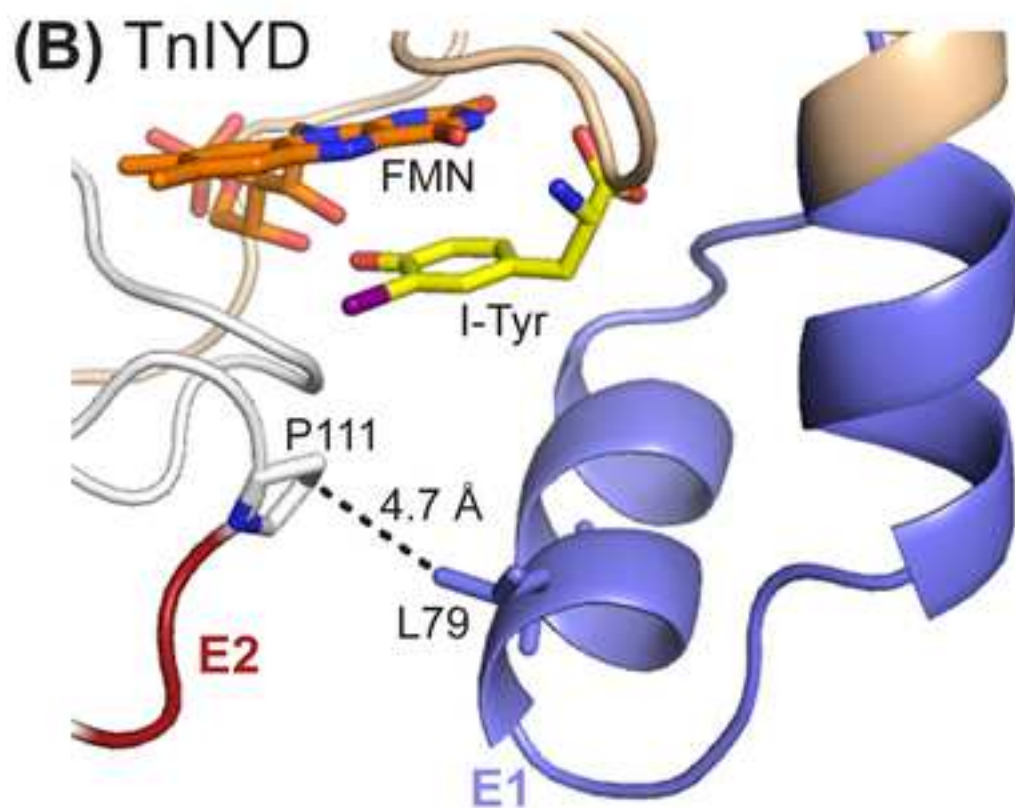
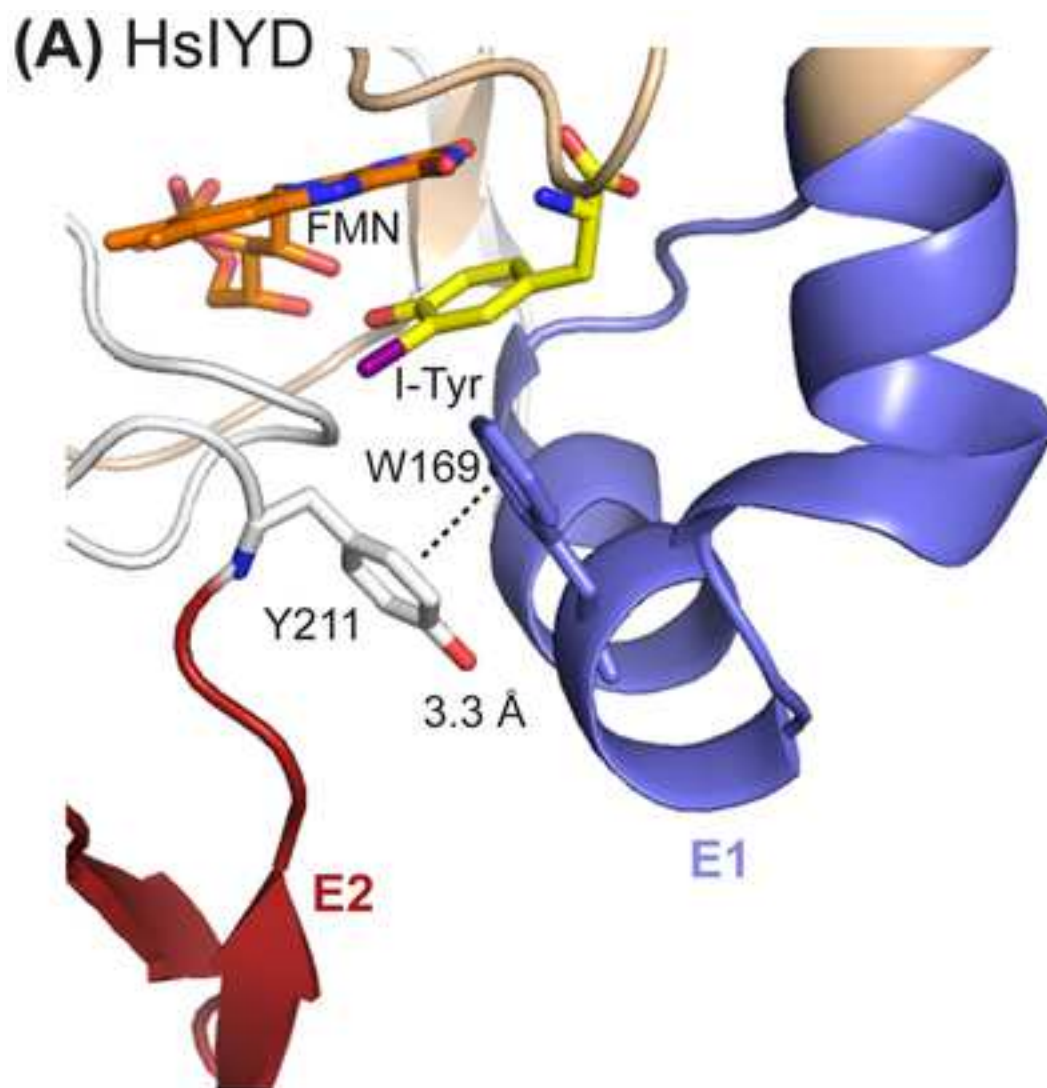
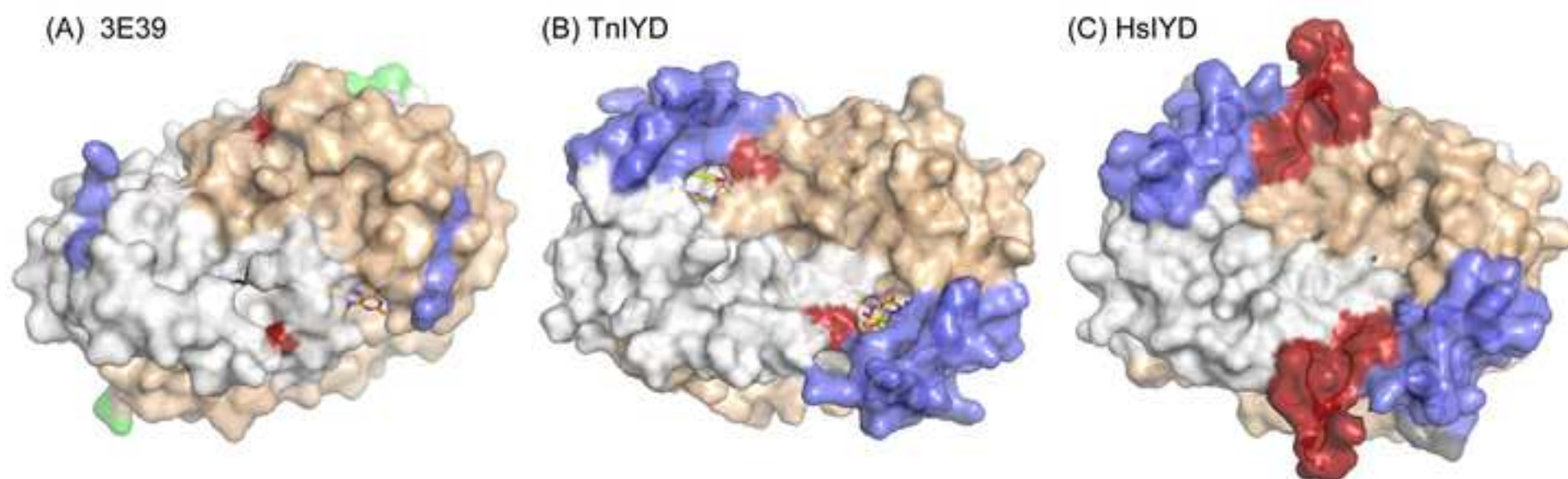


Figure 4





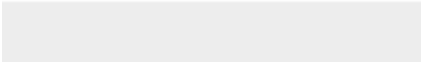






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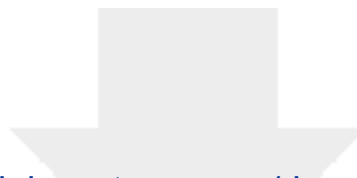
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Author contributions

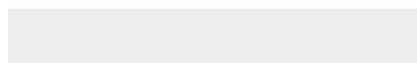
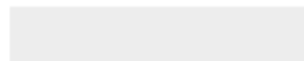
Z.S. performed the cloning, expression and general characterization of TnIYD. Z.S. and B.X. generated and characterized mutants of TnIYD. S.S. measured dehalogenation of 2-IP. Z.S. crystallized the proteins and J.K. solved their structures. S.R. supervised all aspects of the project and all authors discussed the results and contributed to the final presentation.

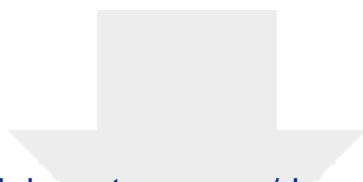
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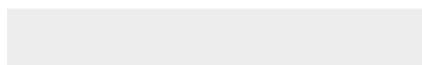
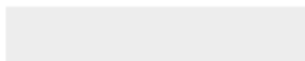
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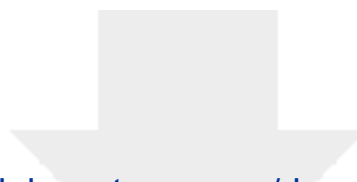




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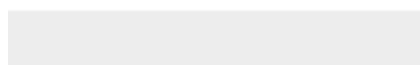
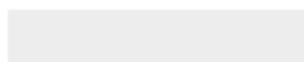
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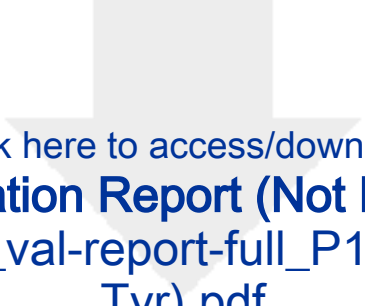




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