Published in final edited form as:

J Am Chem Soc. 2021 March 31; 143(12): 4680–4693. doi:10.1021/jacs.1c00175.

Molecular Rationale for Partitioning between C–H and C–F Bond Activation in Heme-Dependent Tyrosine Hydroxylase

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Abstract

The heme-dependent L-tyrosine hydroxylases (TyrHs) in natural product biosynthesis constitute a new enzyme family in contrast to the nonheme iron enzymes for DOPA production. A representative TyrH exhibits dual reactivity of C–H and C–F bond cleavage when challenged with 3-fluoro-L-tyrosine (3-FTyr) as a substrate. However, little is known about how the enzyme mediates two distinct reactions. Herein, a new TyrH from the thermophilic bacterium *Streptomyces sclerotialis* (SsTyrH) was functionally and structurally characterized. A *de novo* crystal structure of the enzyme–substrate complex at 1.89-Å resolution provides the first comprehensive structural study of this hydroxylase. The binding conformation of L-tyrosine indicates that C–H bond hydroxylation is initiated by electron transfer. Mutagenesis studies confirmed that an active site histidine, His88, participates in catalysis. We also obtained a 1.68-Å resolution crystal structure in complex with the monofluorinated substrate, 3-F-Tyr, which shows one binding conformation but two orientations of the fluorine atom with a ratio of 7:3, revealing that the primary factor of product distribution is the substrate orientation. During *in crystallo* reaction, a ferric-hydroperoxo intermediate (compound 0, Fe³⁺-OOH) was observed with 3-F-Tyr as a substrate based on characteristic spectroscopic features. We determined the crystal structure of

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.1c00175>.

Experimental details, spectroscopic and structural information on TyrH complexes and the hydroperoxo intermediate (PDF)

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/jacs.1c00175>

The authors declare no competing financial interest.

The SsTyrH crystal structures have been deposited in the RCSB Protein Data Bank (<https://www.rcsb.org>) with the PDB codes: 7KQR, 7KQS, 7KQT, and 7KQU. The nucleotide sequence of codon-optimized construct was documented in GenBank with accession number MW418022. The protein sequence was documented in NCBI with the following accession number: WP_051872337.

this compound 0-type intermediate and refined it to 1.58-Å resolution. Collectively, this study provided the first molecular details of the heme-dependent TyrH and determined the primary factor that dictates the partitioning between the dual reactivities of C–H and C–F bond activation.

Graphical Abstract

INTRODUCTION

Histidine-ligated heme-dependent L-tyrosine hydroxylases (TyrH) belong to a new group of microbial enzymes that catalyze the first biosynthetic step of some antibacterial antitumor natural products from *Actinomyces*. Members of this protein group are responsible for hydroxylating L-tyrosine (Tyr) to L-3,4-dihydroxyphenylalanine (DOPA) in the presence of hydrogen peroxide (Figure 1A). LmbB2 is in the biosynthetic pathway of lincomycin and was the first characterized member of this family.^{1–3} In addition to LmbB2, other identified TyrHs and their corresponding natural products include Por14 and porothramycin,⁴ Orf13 and anthramycin,^{5,6} SibU and sibiromycin,⁷ HrmE and hormaomycin,⁸ as well as TomI and tomaymycin.⁹ Previous bioinformatic studies also identified a few putative TyrHs from different *Actinomyces* strains with unknown downstream natural products.^{6,10} One example is found in the thermophilic bacterium *Streptomyces sclerotialis* in which a putative TyrH encoded by gene 2337 was proposed through a genome context analysis,¹⁰ but it has not been functionally characterized.

Heme-dependent hydroxylation is commonly accomplished by enzymes with a cysteine as the proximal axial ligand, as seen in the cytochromes P450 and peroxxygenases. It is known that thiolate ligation of the heme iron enhances hydrogen atom transfer (HAT) and oxygenation, whereas histidine ligation favors one-electron oxidation reactions. Such a difference likely arises from the *trans*-thiolate ligation inducing a more substantial push effect, leading to a more basic oxo group bound with the high-valent heme iron.^{11–13} TyrH is distinct from other heme-dependent hydroxylases due to its use of an axial histidine ligand,^{3,6,14} resulting in an intriguing hydroxylation mechanism.

Our recent mechanistic study on a TyrH protein, LmbB2, demonstrated its substrate promiscuity on Tyr analogs.³ While it is necessary to maintain the 4-hydroxyl group of Tyr,

analogues with ring-deactivating substitutions on the 3-position were able to be hydroxylated. The most interesting observation is that LmbB2 could catalytically cleave both C–H and C–F bonds when confronted with the alternate substrate, 3-fluoro-L-tyrosine (3-F-Tyr).³ In addition to the native C–H bond hydroxylation to generate 3-fluoro-5-hydroxy-L-tyrosine (3-F-5-OH-Tyr), LmbB2 could activate the C–F bond and thus generate an unexpected, defluorinated product, DOPA (Figure 1B). A two-electron difference is expected between the departure of fluoride versus proton from the oxygenated carbon. Therefore, the C–F bond cleavage requires a different mechanism than the C–H bond cleavage. The ability to cleave a C–F bond by TyrH has garnered significant interest and attention;^{15–17} however, the molecular rationale for dual reactivity and catalytic intermediates remain to be elucidated.

The C–F bond is among the most stable chemical bonds, and the biodegradation of fluorinated hydrocarbons, including fluoroarenes and fluoroalkanes, is exceptionally challenging in nature.¹⁸ However, several metalloenzymes have been acknowledged for their ability to perform defluorination, such as histidine-ligated heme-dependent dehaloperoxidase (DHP) and TyrH,^{3,19} thiolate-ligated heme-dependent cytochrome P450,^{20,21} pterin-dependent nonheme tyrosine/phenylalanine hydroxylase,^{22,23} 2-oxoglutarate-dependent nonheme iron enzymes,^{24,25} Rieske dioxygenase, and thiol dioxygenase.^{26–29} In addition to iron-based proteins, a recent finding reported the biocatalytic scission of the robust C–F bond in a copper-containing enzyme, galactose oxidase.³⁰

Compared to the well-characterized DHP, TyrH shows several distinct features. DHP favors *para*-substituted phenols, whereas TyrH favors *meta*-substituted phenols. DHP exhibits a halogen reactivity in the order of Br > Cl > F, whereas TyrH of reactivity is F > Cl > I.³ The differences between the halogenated analogs in chemical property, binding affinity, and steric effect could all contribute to the inversed reactivity tendency, but more importantly, these two classes of enzymes use distinct mechanisms. DHP mediates an oxidative C–X (X represents a halogen) bond cleavage and yields quinone products. In contrast, TyrH promotes a nonoxidative C–X bond cleavage and give rise to catechol products.³

The ability to perform defluorination is largely associated with the structural features of these biocatalysts;¹⁷ however, the structure of heme-dependent TyrH has not been solved. Although we have proposed plausible mechanisms for C–H and C–F bond cleavage by TyrH,^{3,17} without the aid of protein structural information and characterization of intermediates, the chemistry of the TyrH reaction cannot be fully established and compared to other heme-dependent enzymes. On the basis of bioinformatics, TyrH was expected to possess a unique protein fold, as no conserved domains or motifs related to its cofactor binding were found.⁶ As such, determining the protein structure of TyrH and its interaction with the substrate are of great importance in further understanding the unusual reactivity on both C–H and C–F bond activation.

Here, we isolated the putative TyrH from *S. sclerotialis* and defined its hydroxylation activity on the native substrate Tyr and alternate substrate 3-F-Tyr. We then determined the first crystal structures in various catalytically relevant forms, including the Tyr- and 3-F-Tyr-

bound complexes, which illuminate the active site catalytic residues for C–H bond activation and a heme-bound hydroperoxo intermediate in the pathway of defluorination of 3-F-Tyr.

RESULTS

Identification and Characterization of a Thermophilic TyrH.

The crystallization of our previously characterized TyrH protein, LmbB2, was found to be challenging. Hence, we turned our attention to its thermophilic counterparts, as we recently did for LmbB1, a DOPA extradiol dioxygenase. Structural determination of the dioxygenase was achieved via isolated protein from a thermophilic bacterium *S. sclerotialis* (i.e., SsDDO).¹⁰ Gene 2337 attracted our attention, as it encodes a putative TyrH from *S. sclerotialis* (SsTyrH). This putative TyrH shares 43% protein sequence identity with LmbB2 as indicated by pairwise sequence alignment through EMBOSS Needle.³¹ The high sequence similarity between SsTyrH and LmbB2, as well as the thermophilic nature of the source organism, indicate this homolog is likely more amenable to further study, especially for X-ray crystallographic characterization. Hence, gene 2337 was codon-optimized and synthesized. The gene product, a putative SsTyrH, was expressed in *E. coli* and isolated as a soluble protein with an expected molecular weight of 34 kDa. SsTyrH exists predominantly as a monomer in solution as indicated by size-exclusion chromatographic analysis (Figure S1).

SsTyrH exhibited a pronounced Soret band maximum at 403 nm, with absorbance features at 536 nm in the α/β region, and at 502 and 628 nm in the charge transfer (CT) band region (black trace, Figure 1C). The absorbance features are similar to those of other reported TyrH proteins.^{6,14} Upon the addition of Tyr (1 mM), the Soret band decreased with a 1.5 nm redshift of the λ_{max} . A new spectral feature emerged at 570 nm, while the 628 nm feature shifted to 622 nm upon binding Tyr (blue trace, Figure 1C) and a smaller blueshift with 3-F-Tyr as the ligand (red trace). The heme cofactor in SsTyrH showed a high spin ($S = 5/2$), axial EPR signal at $g_{\perp} = 5.82$, $g_{\parallel} = 2.00$ (black trace, Figure 1D). Substrate-binding to the enzyme resulted in a more homogeneous heme environment, as indicated by a slightly sharper high-spin signal with g values of $g_{\perp} = 5.81$, $g_{\parallel} = 2.00$ (blue trace, Figure 1D). Similar UV–vis and EPR spectral changes were also observed with 3-F-Tyr binding (red traces, Figure 1 C, D, Table S1), indicating that 3-F-Tyr binds the active site in a similar conformation as the native substrate. We attempted to investigate the binding behaviors of Tyr and 3-F-Tyr by following the spectral changes at the Soret band or 570 nm through substrate titrations. As shown in Figure S2, SsTyrH exhibited strong binding with both substrates, and the binding constant (K_D) could not be further analyzed using this method because its value is below the minimally required enzyme concentration for analyzing spectral difference, i.e., 10 μM . The binding stoichiometry was in the range of 0.7–0.8 for both substrates, as indicated by the breakpoints. This observation is consistent with the results obtained by isothermal titration calorimetry, which found that LmbB2 has a K_D of 1.35 μM for Tyr and 22.2 μM for 3-F-Tyr.³ Overall, the UV–vis and EPR data suggested that upon substrate binding, the electronic structure of the heme center undergoes relatively minor changes, which is a common feature observed in various TyrHs.^{3,6,14}

Next, we sought to describe the activity of SsTyrH toward Tyr and 3-F-Tyr. Under the previously reported conditions,³ upon reacting with H₂O₂, a majority of Tyr was readily converted to the hydroxylated product, DOPA (Figure 1E). Moreover, SsTyrH was capable of cleaving either the C–H or C–F bonds of 3-F-Tyr. When 3-F-Tyr was introduced as the substrate, it was converted to DOPA and 3-F-5-OH-Tyr (Figure 1F). The proportion of C–H and C–F bond cleaved products, 3-F-5-OH-Tyr and DOPA, was nearly 1.4:1, making SsTyrH more efficient at defluorination than the previously reported TyrH, LmbB2 (ratio of 2:1). Considering the thermophilic nature of this enzyme, we also investigated its optimal pH and temperature. SsTyrH exhibited maximal *in vitro* activity at pH 7.0 and 50 °C (Figure S3). Steady-state kinetic assay of SsTyrH with Tyr gave a K_M of $530 \pm 50 \mu\text{M}$ and a k_{cat} of $14.2 \pm 0.5 \text{ min}^{-1}$ at room temperature. In the case of 3-F-Tyr, the values are $740 \pm 90 \mu\text{M}$ and $4.7 \pm 0.2 \text{ min}^{-1}$ for C–H bond scission, and the kinetic assay of C–F bond scission was fitted to the Hill equation with a V_{max} of $2.6 \pm 0.1 \text{ min}^{-1}$, a K_M of $940 \pm 50 \mu\text{M}$, and a Hill constant of 1.5 ± 0.1 (Figure S4). The determined kinetic parameters of 3-F-Tyr reactions could be higher than the theoretical values since the hydroxylation of C–H and C–F bonds occurred simultaneously; however, it is suggested that 3-F-Tyr has an overall higher K_M than the native substrate. With the fluoro substituent, the C–H bond hydroxylation became 3-fold slower, and the C–F bond hydroxylation is half as efficient as the C–H bond functionalization.

SsTyrH also generated minimal products from both C–H and C–F bond activation in the presence of ascorbate under aerobic conditions (Figure S5), which is consistent with the previous finding from Orf13 that TyrH can utilize oxygen and ascorbate to hydroxylate Tyr, but its catalytic rate was about 100-fold lower than that of the H₂O₂-dependent reaction.⁶ Collectively, SsTyrH was experimentally established as a new member of the TyrH protein group.

de novo Crystal Structure of TyrH in Complex with the Native Substrate.

The tag-free form of SsTyrH was prepared and co-crystallized with Tyr (see Materials and Methods). However, the ligand-free protein was resistant to crystallizing under similar conditions. Since no known protein structures could successfully function as a search model to assist structural determination, the *de novo* crystal structure of SsTyrH was determined by single-wavelength anomalous diffraction (SAD). A crystal of seleno-L-methionine (SeMet)-substituted enzyme–substrate (ES) complex diffracted to 1.98-Å resolution (Table 1). Next, an ES complex structure of wild-type (wt) SsTyrH was obtained from the native protein and refined to 1.89-Å resolution. The crystal structure belongs to the $P2_1$ space group and contains two molecules in an asymmetric unit (Figure 2A). These two subunits structurally resemble each other with a root-mean-square deviation (rmsd) of 0.56 Å over 299 C $_{\alpha}$ atoms. The buried surface area between two subunits is 1427 Å², corresponding to 10% of the total accessible area of one monomer. Since the monomeric state is the predominant form in solution and is functionally active (Figure S1), the observed dimeric structure with a relatively small subunit–subunit interface is possibly caused by crystal packing. SsTyrH is comprised of 314 amino acids, while residues at each terminus (1–5 and 309–314) are disordered in the crystal structure.

Each SsTyrH monomer contains eleven α -helices ($\alpha 1$ to $\alpha 11$), two short β -strands ($\beta 1$ and $\beta 2$), and four 3_{10} helices ($\eta 1$ to $\eta 4$) (Figure 2B). Along with a short two-stranded β -sheet, a helical bundle formed by three long α -helices ($\alpha 2$, $\alpha 3$, and $\alpha 7$) separate the molecule from the center, flanked by other α -helices and 3_{10} helices. Two α -helices ($\alpha 1$ and $\alpha 10$) and a bent α -helix ($\alpha 11$) locate behind the long helical bundle, and a heme-binding pocket surrounded by short helices ($\eta 1$, $\eta 2$, $\alpha 4$, $\alpha 5$, $\alpha 8$, and $\alpha 9$) is in the front. A *b*-type heme is located near the protein surface as indicated by its apparent electron density. The distal pocket of the heme center is shielded and, thus, isolated from bulk solvent. As shown in Figure 2C, His196 from $\alpha 7$ is the proximal ligand of the heme and it coordinates to the iron with the N ϵ . The histidine axial ligand is nearly perpendicular to the heme plane. The heme is further supported by Arg172, Ser157, and Ser 209 through H-bonding interactions with the propionate groups. The axial histidine ligand is strictly conserved among the identified homologs, and other identified heme stabilizing residues are also highly conserved across the TyrH amino acid sequences (Figure S6).

Additional electron density was observed in the active site of the co-crystallized structure, which was well fitted with full occupancy for the substrate, Tyr (Figure 2D). The substrate binds in the distal pocket at a distance of about 9.5 Å from its aromatic ring to the surface of the protein, much deeper than the heme prosthetic group (<3 Å to the protein surface). This observation can be interpreted as a more open conformation present in SsTyrH in the absence of the substrate, which may explain why the substrate-free protein does not crystallize under the same conditions. The Tyr substrate was found to be stabilized by extensive interactions with second-coordination sphere residues. The carboxylate group of the substrate is anchored by the guanidinium moiety of Arg146 through salt bridges, and it also H-bonds with the phenol of Tyr141 and the main chain of Leu210. The substrate's amine group does not directly interact with protein residues, but it forms indirect interactions through ordered solvent molecules. His88 and Tyr230 anchor the 4-hydroxyl of the substrate from the opposite side relative to the heme, sequestering the 4-hydroxyl away from the iron center. These second-coordination sphere residues involved in substrate binding are strictly conserved among all TyrH homologs (Figure S6). The plane of the aromatic ring of the bound substrate is tilted toward the porphyrin ring at roughly 45° and is oriented toward the iron ion at a distance of 5 Å. On the basis of the structural refinement, a water molecule is located above the porphyrin ring, 3.1–3.2 Å from the iron ion, which is too distant to directly coordinate to heme and is consistent with the high-spin species characterized by EPR spectroscopy of the binary complex in solution.

Identification of the Second-Coordinate Sphere Histidine as an Essential Catalytic Component.

We have demonstrated that the 4-hydroxyl group of the substrate is critical for the TyrH-mediated reactions.³ The ES complex structure obtained in this work reveals that the 4-hydroxyl group interacts with two strictly conserved residues, His88 and Tyr230 (Figure 2D). Hence, we investigated the roles of these residues in catalysis with a site-directed mutagenesis study. The catalytic activities of the functional group-deleted variants, H88A and Y230F, were examined. At the optimal temperature and pH, SsTyrH exhibited a specific activity of 640 ± 20 nmol min⁻¹·mg⁻¹ with the native substrate. Under typical activity assay

conditions with Tyr as the substrate, Y230F retained the hydroxylation ability with an activity of $580 \pm 10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, while no hydroxylation activity was observed for H88A (Figure 3A). We further generated H88Y, Y230H, and a double-site variant H88Y/Y230H to examine whether the swapped position would cause any difference. The results showed that Y230H was still active but formed less product, while H88Y showed no observable activity as H88A (Table 2), suggesting His88 is critical for catalysis but not Tyr230. Interestingly, the double-site variant H88Y/Y230H with the swapped position of the His and Tyr generated a small amount of DOPA (front trace, Figure 3A), accounting for 10% of the product generated by the wt enzyme, suggesting the positioning of His88 affects the catalytic efficiency.

The specific activities of SsTyrH on 3-F-Tyr were determined to be 205 ± 9 and $102 \pm 3 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for C–H and C–F bond cleavages, respectively. We studied the above protein variants with 3-F-Tyr as the primary substrate (Figure 3B). Similarly, Y230F and Y230H exhibited dual reactivity of C–H and C–F bond cleavage as wt enzyme (Table 2). Again, no catalytic activity was observed for H88A and H88Y with the alternate substrate 3-F-Tyr. A trace amount of 3-F-5-OH-Tyr was observed for the double-site variant, also accounting for approximately 10% hydroxylation activity of the wt enzyme, but no C–F bond cleavage product DOPA was detected. Collectively, these results indicate that His88 is essential for tyrosine hydroxylation, while Tyr230 has a marginal impact on catalysis.

Next, we characterized the His88 and Tyr230 variants using UV–vis and EPR spectroscopies. As expected, Tyr230 variants retained the spectroscopic features of wt SsTyrH, with similar λ_{max} of Soret and Q/CT bands (Figure 3C) as well as the g values and line shape in their EPR spectra (Figure 3D). Upon substrate binding, the Tyr230 variants also exhibited similar spectral changes as observed in wt SsTyrH (Figure S7A,B). In contrast, His88 variants showed noticeable spectroscopic differences compared to the native protein. The Soret bands redshifted to 411 nm for both H88A and H88Y variants, and new features originated in α/β region for H88A and H88Y (Figure 3C). The double-site variant H88Y/Y230H also exhibited a distinct absorption spectrum with the Soret band maximum at 408 nm. Upon substrate addition, the intensity of the Soret band of the His88 and double-site variants underwent a slight decrease but no significant changes in the α/β region (Figure S7 C–E). The feature at 570 nm in wt and Tyr230 variants, associated with substrate addition, was not observed in His88 variants, which indicates that the 570 nm feature is a characteristic spectral signature of the catalytically relevant ES complex. Substrate titration experiments were also performed on Y230F and Y230H variants. As shown in Figure S2, Y230F variant bound substrate too tightly to yield a binding constant using this method, while Y230H variant showed relatively weak binding with a K_D of $109 \pm 5 \mu\text{M}$. This observation presumably explains the results of specific activities that Y230F had a similar hydroxylation activity as wt enzyme, but Y230H was relatively less active. The EPR spectra of substrate-free His88 and double-site variants showed an inhomogeneous heme center. In addition to the axial high-spin species at $g = 5.8$, multiple unresolved low-spin resonances were observed with central g values of 2.27 and 2.00 (Figure 3D). Upon substrate addition, the high-spin species of H88A and H88Y had no significant changes, while the H88Y/Y230H variant showed a similar EPR spectrum as observed in the wt SsTyrH (Figure S7). The low-spin species had no measurable spectral change upon substrate addition; hence,

they may represent a catalytically inactive species due to the alteration of His88. The UV-vis and EPR spectral features of these variants are summarized in Table S1. The significant spectral characters of the His88 variants revealed that alteration of His88 caused a reorganization of the distal pocket, and hence, perturbed heme environment and electronic properties. Such perturbation is observed in double-site variant to a lesser extent, suggesting that the histidine preservation could partially rescue the catalytic function even though its position is altered in the swapped double mutant. In the ES complex structure, His88 is stabilized by the backbone carbonyl group of Trp84, and it forms a π - π stack with Phe234. No other residues were found to form a proton shuttle or hydrogen bonding network (Figure S8). Overall, His88 plays a crucial role in catalysis, while Tyr230 assists substrate binding and is not critical for the chemistry.

Two Binding Orientations of 3-Fluoro-L-Tyrosine in the Distal Pocket.

To interrogate the mechanism of C-H and C-F bond activation of TyrH, 3-F-Tyr was co-crystallized with SsTyrH. We obtained a structure of the binary complex and refined it to 1.68-Å resolution (Table 1). The overall structure of the ES complex with 3-F-Tyr as the substrate has no substantial difference from the Tyr-bound complex structure, showing a rmsd value of 0.25 Å over 601 C $_{\alpha}$ atoms of the dimer. As revealed by electron density maps, 3-F-Tyr binds to the enzyme in the same conformation as Tyr, with well overlapped phenolic and main chain moieties. However, when no substituent on the phenyl ring of the Tyr was modeled, a strong positive electron density corresponding to a fluorine atom was observed on one of the *ortho* positions with a minor positive density on the other side (Figure S9A). The initial attempt to fit 3-F-Tyr with a single orientation at full occupancy was not successful. Placing the fluorine substituent at either side resulted in negative electron density maps, and the position without fluorine substitution showed positive density in the F_0-F_c maps (Figure S9B,C). Next, two orientations of 3-F-Tyr were invoked to fit the electron density with unrestrained occupancy during refinement. As shown in Figure 4A (top view) and Figure S9D (side view), modeling of the electron density with two orientations resulted in no residual density maps on either side of the aromatic ring. The final structure reveals the two orientation occupancies to be 0.7 and 0.3, corresponding to the fluorine substituent facing upward (orientation A, Figure 4B) and downward (orientation B, Figure 4C) to the heme porphyrin plane, respectively. The 7:3 occupancy ratio is also applicable to other 3-F-Tyr crystal structures, *vide infra*. The 4-hydroxyl positioning is only slightly different between these two conformations. At the same time, the rest of the amino acid moieties nearly overlap, indicating that 3-F-Tyr binds to TyrH in a single binding mode but with restricted ring rotation resulting in two preferred orientations. Overall, both orientations bind similarly as native substrate, Tyr. In orientation A, the fluorine substituent interacts with His88, while in orientation B it interacts with the main chain carbonyl of Ser157. The structural determination of two binding orientations in one conformation in the distal pocket of the heme revealed that the partitioning of the C-H/C-F bond cleavage reactions of 3-F-Tyr is dictated by the substrate orientation.

Ferric Heme-Bound Hydroperoxo Intermediate Captured during *in crystallo* Reaction.

The *in crystallo* chemical reaction was performed to accumulate possible catalytic intermediates, as reactions are significantly slowed in the crystalline state compared to

aqueous reactions.³² The crystals of the 3-F-Tyr-bound SsTyrH complex were soaked in hydrogen peroxide-containing mother liquor for various time intervals. After 20-s of reaction, the 3-F-Tyr bound co-crystal showed a single-crystal UV-vis absorption spectrum distinct from the unreacted crystals. As shown in Figure 5A (black trace), the 3-F-Tyr-bound binary complex crystal showed a Soret peak maximum at 400 nm and other features at 499, 532, 570, 626 nm, which were in line with absorption spectra obtained in the solution state. The slight differences of λ_{max} between crystalline and solution states are anticipated, resulting from crystal packing and pH difference as reported in a nonheme dioxygenase study.³² In addition, the absorbance of Soret peaks was easily saturated due to the dense packing of crystals, hence the readings of λ_{max} based on the peak shape could introduce some inaccuracy. After reacting with H_2O_2 , the Soret band red-shifted to 409 nm, and new features at 360, 525, and 554 nm emerged concomitant with the disappearance of the original binary complex spectral signatures (red trace, Figure 5A). This single-crystal spectrum, especially the prominent features generated at the Q-band region, is in agreement with the reported absorption spectra of ferric-bound hydroperoxo characterized in other heme proteins.^{33–37} In particular, histidine-ligated ferric-hydroperoxo species in horseradish peroxidase generated through radiolytic reduction shows spectral signatures of a Soret band at 420 nm and sharp band maximum at 557 nm;³³ such a ferric-hydroperoxo intermediate was also reported in histidine-ligated heme oxygenase with absorption spectra maxima at 421, 530, and 557 nm.³⁷ Notably, the single-crystal UV-vis spectra were recorded before X-ray exposure to eliminate possible photo-reduction. Additionally, the absorption spectra of chemically reduced TyrH in complex with 3-F-Tyr were also obtained, in both solution and crystalline states, for comparison, which showed significant differences in both Soret and α/β regions from the spectra of ferric-hydroperoxo species (Figure S10). On the basis of the absorption spectral features, the binary complex crystals reacted with H_2O_2 for 20 s yielded an intermediate corresponding to a ferric-hydroperoxo, also known as compound 0 (Cpd 0, $\text{Fe}^{3+}\text{-OOH}$).

The X-ray diffraction data of the crystals with spectral signatures resembling ferric-hydroperoxo were then collected, and the data set with the best resolution was refined to 1.58 Å (Table 1). When 3-F-Tyr was fitted with two orientations, an extra ellipsoid electron density was found above the heme iron which was ideal for accommodating a diatomic molecule (Figure 6A,B). The modeling of one single oxygen atom to the excess electron density resulted in positive electron density above the iron ion; fitting with a hydroperoxo molecule with full occupancy resulted in slight negative electron density, while a 0.8 occupancy yielded the best fit with no additional residual density observed (Figure S11). Together with the absorption spectrum, this intermediate was identified as a ferric-hydroperoxo intermediate. In this structure, the Fe–O distance is 2.2 Å, the O–O bond length is 1.4 Å, and the angle of Fe–O–O is 138° in one subunit and 2.4, 1.4, and 128° in another subunit (Table S2). Additionally, both oxygen atoms of the hydroperoxo are stabilized by the primary amine of the substrate, and the distal oxygen is further stabilized by the backbone of Gly158 and one of the heme propionate groups. The presence of the H-bonding interaction with a deprotonated carboxylate group suggests that the distal oxygen is in a protonated state as hydroperoxo rather than peroxo dianion. The overall structure has minimal changes compared to the initial 3-F-Tyr-bound binary complex structure, with rmsd value of 0.17 Å

over 600 C_α atoms. Fitting with two oxygen atoms to the electron density in an end-on mode can be assigned to other commonly observed heme adducts, such as ferric-superoxo and ferrous-dioxygen. However, these adducts in histidine-ligated heme proteins have quite different optical spectra in both Soret and Q/CT band regions from the ferric-hydroperoxo reported here, which helps to rule out these possibilities.^{38–41}

In contrast, the Tyr-bound binary complex crystals showed no signs of such a hydroperoxo intermediate under the same reaction conditions. After reacting with H₂O₂ for 20 s, the Tyr-bound crystals exhibited single-crystal UV–vis spectral features almost identical to the unreacted, binary complex crystals (orange trace, Figure 5A). A possible reason could be that the hydroperoxo decays faster than its formation and would not accumulate. However, with the alternate substrate 3-F-Tyr, the presence of a fluorine substituent deactivates the aromatic ring and lowers the pK_a of the 4-OH group. These effects potentially increase the half-life of the ferric-hydroperoxo, allowing it to be captured during *in crystallo* reactions. The spectroscopic and structural characterization of a ferric-hydroperoxo intermediate specific to the C–F bond cleavage is consistent with our previous isotope-labeling study of the TyrH reaction, wherein a significant isotope scrambling was observed in reactions of 3-F-Tyr with 18O-labeled water, indicating substrate-based intermediates or iron-bound oxidants are longer-lived.³ Cpd 0 characterized in this study could be a candidate for one of the iron-bound oxidants.

Next, we generated an unreactive ternary complex for comparison. We soaked sodium cyanide into the 3-F-Tyr/SsTyrH co-crystals to compare the cyano ternary complex structure with the ferric-bound hydroperoxo intermediate. The Soret band of the cyanide-soaked crystal redshifted to 412 nm concomitant with the appearance of a shoulder centered at 365 nm, and the spectral features in the α/β region became unresolved with features centered at 535 and 560 nm (Figure 5B). These spectral changes observed *in crystallo* are consistent with the solution data of SsTyrH bound with 3-F-Tyr and cyanide, which exhibited absorbance features at 414, 538, and 563 nm (Figure S12). The crystal structure of SsTyrH complexed with cyanide and 3-F-Tyr was determined at a resolution of 1.84 Å (Table 1). Superposition of this unreactive ternary structure with the reactive Cpd 0 intermediate results in a rmsd of 0.22 Å over 602 C_α atoms. Fitting the cyanide molecule to the electron density enabled us to visualize the cyanide as an axial ligand that coordinates the iron with a C–Fe distance of 2.0 Å, and an Fe–C≡N angle of 153° (Figure 6C). The distance and angle between iron and cyanide are 1.9 Å and 167° in the second subunit (Table S2). Cyanide also interacts with the substrate amine group and the backbone amide of Gly158. It is worth mentioning that one of the propionate groups is originally supported by Ser157 and Ser209 in the binary complexes and stabilizes the hydroperoxo molecule of Cpd 0. The propionate group is less ordered in the cyano structure and no longer forms interactions with either protein residues or the cyano ligand. A noticeable ruffling of the porphyrin ring was also observed in the cyano structure. These local discrepancies are likely caused by soaking cyanide at a higher pH over a significant period (pH 10, 2 h). Additionally, cyanide as an axial ligand binds to heme in a more linear manner than hydroperoxo, which results in similar distances from the nitrogen atom of cyanide to the *ortho* carbons of 3-F-Tyr in both binding orientations. Such a binding conformation is not informative regarding which binding orientation is responsible for C–H or C–F bond cleavage. Therefore, the differences

between the reactive and unreactive ternary complex structures are noticeable. Although cyano complex structures are historically used to mimic the oxidant bound ternary complexes in catalytic pathways of heme enzymes, a peroxide bound intermediate is in demand to properly understand the mechanism of TyrH.

DISCUSSION

Structure–Function Relationships in TyrH.

In the *de novo* ES complex structure, the aromatic ring of Tyr is oriented toward the heme at a distance of 5 Å, which is in an ideal conformation for direct electron transfer (ET) with a heme-based oxidant (Figure 7A). A possible candidate for such a heme-based oxidant is Cpd I, a ferryl-oxo coupled porphyrin cation radical, which is commonly invoked as the primary reactive intermediate in heme enzymes and promotes a wide spectrum of oxidative chemistries.^{42,43} In the case of His ligated heme enzymes, Cpd I is thought to prefer ET rather than hydrogen atom transfer (HAT) due to the low pK_a of the corresponding compound II (Cpd II, a ferryl-oxo with a neutral porphyrin).⁴⁴ A known example is DHP, which utilizes ET to activate aromatic substrates through peroxxygenase-like or peroxidase-based mechanism.^{45,46} The substrate binding conformation in TyrH shown in this work unambiguously excludes the possibility of HAT from either 4-hydroxyl or aromatic *ortho* carbons. The 4-OH of the substrate points to the δ edge of heme, away from the iron ion with a distance of nearly 6 Å (Figure 7B); while the distances from two *ortho* carbons to the iron center are 4.9 and 5.7 Å (Figure 7C). Typically, the distance between the oxidant and the hydrogen atom to be abstracted is expected to be within 3 Å, and the hydrogen atom should be directed to the oxidant.^{47,48} Therefore, based on the crystal structures, an ET mechanism is proposed for TyrH as the initial step to activate the substrate.

Although not directly involved in aromatic activation, the 4-OH group in substrate is essential in terms of catalysis.³ Substrate analogs including phenylalanine and *O*-methyltyrosine bind the enzyme, but with no reactivity.^{3,6} The necessity of a 4-OH excludes the possibility of direct oxygen insertion from Cpd I into the aromatic ring to form a Meisenheimer complex as proposed in P450 chemistry.⁴⁹ Furthermore, previous isotope labeling experiments using ¹⁸O-labeled peroxide show that the oxygen source for hydroxylation is H₂O₂ based on the detection of both ¹⁸O-labeled DOPA and 3-F-5-OH-Tyr as predominant products. The formation of doubly labeled products when ¹⁸O-labeled water was used indicates that the aromaticity of substrate is broken at an intermediary state and thus, the 4-OH is activated to exchange with bulk water.³ The structure-directed mutagenesis analysis in this study indicates that His88 is involved in such an activation of the 4-OH group and further demonstrates the importance of the 4-OH group during catalysis.

Collectively, a native C–H bond hydroxylation mechanism could be proposed. As shown in pathway A of Figure 8, an ET promoted by Cpd I yields a tyrosine cation radical with a dramatically decreased pK_a of 4-hydroxyl.⁵⁰ His88-assisted deprotonation generates a quinone radical, which is attacked by Cpd II to generate a heme-bound tetrahedral complex. Since the ET step is fast and reversible, the deprotonation by His88 could be a critical step of the catalysis. Without such an active site base, the reaction does not proceed forward, which explains why the product was not formed in His88 variants. Heterolytic cleavage of

the Fe–O bond, rearomatization, and protonation facilitated by His88 lead to the formation of the final catechol product and regenerate the resting ferric heme. His88 is an essential second coordination sphere residue playing multiple functions in TyrH, including protecting the heme center from forming inactive low-spin species, assisting in substrate binding, and functioning as a general catalytic acid/base. The precise roles of this active site residue will be further investigated in future studies.

Compound 0 as a Common Intermediate with Disparate Outcomes.

Due to their reactive nature, protein-bound ferric-hydroperoxo/peroxo intermediates are rarely characterized in catalytic processes. Historically, such intermediates were generated by the cryoradiolytic reduction of oxy-ferrous complexes which has been successfully used in systems such as myoglobin,^{40,51,52} chloroperoxidase,^{34,36} horseradish peroxidase,³³ heme oxygenase,³⁷ etc. In this study, we took advantage of the significantly altered kinetics *in crystallo* to trap a longer-lived hydroperoxo intermediate associated with C–F bond activation, resulting in a 1.58-Å resolution crystal structure and single-crystal spectroscopic signatures. For comparison, we summarized other structurally characterized ferric-hydroperoxo/peroxo intermediates. As shown in Table 3, the bond length and coordination angle vary with different proximal ligands, and for histidine-ligated heme proteins characterized at high resolutions, these parameters are generally reported with Fe–O bond of 1.9–2.5 Å, O–O bond of 1.3–1.6 Å, and Fe–O–O angle of 120–135°. The values of the TyrH intermediate reported in this study fall into these ranges. The absence of such an intermediate in native reactions implies that its population could benefit from stabilization by the fluorine substitution.

The 3-F-Tyr bound complex shows the same substrate binding conformation as that in the Tyr-bound complex, but with two fluoro orientations, which indicates that the precise substituent orientation is critical for dictating the oxidation outcome and explains the dual reactivity of C–H/C–F bond activation. This is consistent with our previous mechanistic investigations which found that C–H and C–F bond cleavages proceed through independent pathways. Substrate consumption and formation of the two products were found to be linearly dependent on H₂O₂ concentration, demonstrating that the reactions are independent (not processive) and that a single pathway could not yield both products.³ The C–H bond and C–F bond cleavage reactions also differ by two electrons (F[–] vs H⁺ as a leaving group from the oxygenated carbon). The additional electrons for C–F bond cleavage are supplied by the oxidation of a second equivalent of H₂O₂ to O₂.³ In order to balance each overall chemical reaction, different iron–oxygen species are required to react with the organic substrate. Additionally, C–H bond hydroxylation follows Michaelis–Menten type kinetics, while C–F bond activation requires fitting with the Hill equation, which also supports the C–F bond cleavage proceeding with a distinct mechanism. The apparent cooperativity may arise from the requirement of 3-FTyr to bind before H₂O₂ in order to proceed with C–F bond activation, while ordered binding may not be necessary for the native reaction.

In the ferric-hydroperoxo structure, the distal oxygen of the peroxo is 3.9 and 4.6 Å away from two *ortho* carbons (referred to as C3 and C5) in both orientations, which are too distant to form a direct interaction (Figure S13). Hence, an active site reorganization is anticipated

in order to lead the reaction to a productive trajectory. Since the C–H bond activation pathway shows a faster rate and yields more product than the defluorination reaction, the specific binding orientation with more population, i.e., orientation A, is likely to proceed C–H bond hydroxylation. We speculate that C5 of 3-F-Tyr (the unsubstituted *ortho* carbon) in orientation A triggers C–H bond hydroxylation via Cpd I, while C3 of 3-F-Tyr (the fluorinated *ortho* carbon) in orientation B is responsible for defluorination through a ferric-hydroperoxo intermediate. As a result, the catalytic pathway branches from the binding of 3-F-Tyr (Figure 8). Pathway A corresponds to C–H bond cleavage for orientation A, identical to hydroxylation of the native substrate Tyr; pathway B for C–F bond cleavage with orientation B. The ferric-hydroperoxo is responsible for C–F bond activation by performing a nucleophilic attack on 3-F-Tyr of orientation B, which forms a catechol-like intermediate and Cpd I. Thus, the C–F bond activation and the subsequent hydroxylation in TyrH is a nonoxidative process, which is in sharp contrast to the oxidative C–F bond activation described for DHP.^{53,54} Fluoride elimination generates the re-aromatized product, DOPA, and an additional equivalent of peroxide returns the enzyme to its resting state via catalase activity.

TyrH in the Context of Other His-Ligated Heme-Dependent Oxygenases.

With the current protein structure database, tryptophan 2,3-dioxygenase (TDO) is the most structurally related enzyme to TyrH, although they share less than 20% protein sequence identity.⁵⁵ TDO belongs to a histidine-ligated heme dioxygenase superfamily, oxidizing tryptophan or tryptophan-derived substrates with a ferrous heme and molecular oxygen. Indoleamine 2,3-dioxygenase (IDO), PrnB, and MarE are also members of this enzyme family, and the former two have been structurally characterized.^{56–58} Recently, SfmD as a 3-methyl-L-tyrosine hydroxylase involved in the biosynthesis of saframycin has been identified and structurally resembles the TDO superfamily enzymes.⁵⁹ Despite the conservation of the global tertiary fold, the overall rmsd values upon superposition of the structures are strikingly poor due to diverse structural topology. Hence, the deficiency of structural homology observed among these enzymes precludes the possibility of using molecular replacement methods to solve the structure of TyrH or *vice versa*.

Interestingly, although TyrH and the members of the TDO superfamily perform different chemistries on various substrates, their heme prosthetic groups are found to be overlapped in superposed ES complex structures. Surprisingly, even though TyrH and SfmD catalyze the same Tyr-based, *ortho*-hydroxylation, they have distinct heme cofactors, binding domains, and relative positions (Figure S14). These enzymes may be related in an evolutionary perspective, considering TyrH and SfmD both exhibit slow oxygen-dependent hydroxylation activities in the presence of ascorbate,^{6,59} while the TDO superfamily members can exhibit monooxygenation reactivity.^{58,60} We expect to explore more biochemical implications of these related enzymes in the near future and thus, better understand the evolution and structure–function relationships among these histidine-ligated heme enzymes catalysing aromatic amino acid oxygenation reactions.

CONCLUSION

Herein, the TyrH from *S. sclerotialis* is identified as a new member of the heme-dependent tyrosine hydroxylase group. For the first time, the protein structures of TyrH in various forms, including a high-resolution hydroperoxo intermediate, are determined. Together with the biochemical results described in our previous study,³ the observation of dual binding orientations and ternary complexes lead to the proposed mechanism that Cpd I and ferric-hydroperoxo promote C–H and C–F bond cleavages, respectively, which adds another example of metalloenzyme-catalyzed defluorination and offers a promising template in the design and development of new catalytic functions.

MATERIAL AND METHODS

Protein Overexpression and Purification.

The synthetic gene of the codon-optimized SsTyrH from *S. sclerotialis* was cloned into the pET28a-TEV expression vector (GenScript). The expression plasmid of N-terminally His₆-tagged SsTyrH was then transformed into the *E. coli* BL21 (DE3) cells (Merck), cultured in Luria–Bertani medium with kanamycin (50 µg/mL) at 37 °C. For *de novo* structural determination, SeMet-substituted protein was cultured in M9 minimal medium according to the published method.¹⁰ The protein purification was conducted following the previously reported procedure.³ The eluted protein was concentrated using an Amicon centrifugal filter with a 10-kDa cutoff (Millipore) and desalted into 50 mM Tris-HCl and 50 mM NaCl at pH 8.0 for further use. TyrH purified here had a heme occupancy of 80%, and protein concentration was determined based on heme-bound fraction.

SsTyrH variants were generated by PCR using Phusion High-Fidelity PCR Kit (Thermo Scientific). The sequences of the mutagenesis primers are listed in the Supporting Information. The expression and purification for all variants were the same as the wild-type SsTyrH. The His₆-tag was removed for crystallization purposes. The tagged and untagged proteins behaved identically in the spectroscopic and activity studies.

UV–vis and EPR Spectroscopies.

Absorption spectra SsTyrH or variants (10–15 µM) were measured in 50 mM Tris-HCl and 50 mM NaCl at pH 8.0. The ES complexes were prepared by mixing proteins with 1 mM L-tyrosine (99%, Alfa Aesar) or 3-F-Tyr (98%, TCI). Spectra were recorded in a quartz cuvette using a Lambda 25 spectrophotometer (PerkinElmer). Details on *K_D* measurements of Tyr and 3-F-Tyr using UV–vis spectroscopic titration were recorded in Supporting Information.

EPR samples were prepared with SsTyrH or variants (250 µM) in 50 mM Tris-HCl and 50 mM NaCl at pH 8.0. The primary substrate was added at a final concentration of 1 mM. All samples were frozen in 4 mm quartz EPR tubes by liquid nitrogen. X-band continuous-wave EPR spectra were recorded using a Bruker E560 spectrometer at 9.4 GHz microwave frequency with an SHQE high-Q resonator at 100 kHz modulation frequency equipped with a cryogen-free 4 K temperature system as previously described.³ The EPR spectra were collected at 10 K with a microwave power of 1.0 mW. The *g* values reported were obtained by inspection of the EPR line shape.

Activity Assay.

The initial activity examination of SsTyrH and variants (results shown in Figures 2 and 3) were conducted under the reported conditions,^{3,6} at room temperature in 100 mM potassium phosphate with 50 mM NaCl and pH 8.0. SsTyrH or variants (100 μ M) was premixed with either Tyr or 3-F-Tyr (3 mM) for 5 min before H₂O₂ addition (3 mM). A stock of 20 mM H₂O₂ solution was titrated to the enzyme precomplexed with a primary substrate by adding 10 aliquots within 10 min (one addition per min) to minimize heme bleaching. Then 10 μ L of concentrated HCl (6 M) was added to quench the reaction. The final volume was 200 μ L. To determine the optimal pH and temperature, steady-state kinetic parameters, oxygen-dependent activity, and compare the specific activity of SsTyrH and variants, assay conditions were varied (see Supporting Information for details). After the precipitant was removed by centrifugation, the supernatant was filtered using a 10-kDa molecular weight cutoff centrifugal filter (Millipore). A 10- μ L portion of filtrate was injected into an InertSustain C18 column (5 μ m particle size, 4.6 $\times$ 100 mm, GL Sciences Inc.) with a flow rate of 1 mL/min, and then analyzed by a Thermo Scientific Ultimate-3000SD HPLC rapid separation system equipped with a photodiode array detector. The chromatograms were recorded with a full range wavelength from 190 to 800 nm. HPLC profiles presented in this study were chromatograms at 280 nm. The solvent used for isocratic elution was HPLC-grade water with 3% acetonitrile and 0.1% formic acid.

Protein Crystallization.

With crystallization screening (Hampton Research), diffractive crystals of SsTyrH can only be obtained by cocrystallization with a substrate, either Tyr or 3-F-Tyr. His-tagged TyrH were treated overnight with TEV protease in 50 mM Tris-HCl and 50 mM NaCl (pH 8.0) at 4 °C. A HisTrap column followed by a Superdex-75 column (GE Healthcare) was used to further purify the untagged protein. The untagged SsTyrH was prepared in 50 mM Tris-HCl buffer of pH 7.0 and concentrated to 40 mg/mL. The concentrated protein was supplemented with either Tyr (2 mM) or 3-F-Tyr (3 mM) to form ES complex. Then, the ES complex was mixed at a 1:1 volume ratio with a crystallization buffer of 0.1 M Bis-Tris (pH 6.1), 0.2 M MgCl₂, and 16% (w/v) PEG 3350 using the hanging drop, vapor-diffusion method at 289 K. Red, flake shape crystals were formed out of precipitation after 4 days and grew to an optimal size suitable for X-ray diffraction after approximately 1 week. Crystals were cryoprotected with crystallization buffer containing an additional 25% (v/v) glycerol and then flash-cooled in liquid nitrogen.

Preparation of the Unreactive/Reactive Ternary Complex/Intermediate.

To obtain crystals of the cyano ternary complex, crystals of SsTyrH in complex with 3-F-Tyr were soaked in the crystallization mother liquor of pH 10, with added sodium cyanide (40 mM) in a fume hood for 2 h, and then cryoprotected by 25% (v/v) glycerol prior to immersion in liquid nitrogen. To pursue *in crystallo* reaction intermediates, the co-crystallized crystals were incubated with the mother liquor supplemented with H₂O₂ (5mM) and then flash-cooled directly in liquid nitrogen after being dipped into the mother liquor containing 25% glycerol. The reaction intermediate was identified by single-crystal absorption spectroscopy prior to exposure to X-rays for structural determination. Data

collection and structural refinement are described in the Supporting Information. The data collection and refinement statistics are summarized in Table 1. Subunit A was used to make representative figures in this study, and the differences in heme coordination between two subunits are listed in Table S2.

Single-Crystal X-ray Absorption Microspectroscopy.

In situ single-crystal UV–vis absorption spectroscopy was used to characterize the crystals of cyano complex and the ferric-hydroperoxo intermediate. Spectra were recorded at 100 K before X-ray exposure using the microspectrophotometer at SSRL 9–2. Dark and reference spectra were taken in the absence of crystal samples. To minimize the artifacts of frozen cryoprotectant, ice, or the nylon loop, different orientations of a single crystal were scanned by ϕ -angle rotation mode from 0 to 360° with a step of 10°. The optimal absorption spectra with reproducible spectral features were selected to present.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was generously supported by the National Institutes of Health grant GM108988 and the Lutch Brown Endowment fund (to A.L.) We thank the synchrotron beamline 19-BM of the Structural Biology Center (SBC) at the Advanced Photon Source of Argonne National Laboratory (user program GUP 65040) and beamline BL9-2 of the Stanford Synchrotron Radiation Lightsource (SSRL) (user program 5B14), SLAC National Accelerator Laboratory. SBC-CAT is operated by UChicago Argonne, LLC, for the U.S. Department of Energy, Office of Biological and Environmental Research, under Contract DE-AC02-06CH11357. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract DE-AC02-76SF00515.

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Figure 1.

Identification of SsTyrH as a member of the heme-dependent tyrosine hydroxylase group. (A) TyrH oxidizes L-tyrosine (Tyr) to DOPA. (B) TyrH oxidizes 3-F-Tyr to DOPA and 3-F-5-OH-Tyr. (C) UV-vis absorption spectra of ligand-free SsTyrH (black), SsTyrH complexed with Tyr (blue), and SsTyrH complexed with 3-F-Tyr (red). Inset shows Q-band features magnified by 10-fold. (D) X-band EPR spectra of SsTyrH (black), SsTyrH complexed with Tyr (blue), and SsTyrH complexed with 3-F-Tyr (red). (E) HPLC profiles of SsTyrH reaction with Tyr in the absence (gray) and presence (black) of H_2O_2 . (F) HPLC profiles of SsTyrH reaction with 3-F-Tyr in the absence (gray) and presence (black) of H_2O_2 .

Figure 2.

Three-dimensional structure of the SsTyrH ES complex. (A) Two monomers in an asymmetric unit are shown in blue and green, with their heme prosthetic groups colored in dark red. (B) A monomer structure colored by a rainbow spectrum (N-terminus, blue; C-terminus, red). The molecule is composed of 11 α -helices, two β -strands and four 3_{10} helices. (C) A closed surface shows the distal heme pocket in a side view and (D) a top view of the active site with the electron density of the bound substrate. The residue of Leu210 is omitted for clarity. Color code of atoms: carbon, protein, white; carbon, substrate, yellow; carbon, heme, deep red; nitrogen, blue; oxygen, red. The $F_o - F_c$ omit maps are contoured at 3σ , colored in gray and blue for heme and Tyr, respectively. Gray dashed lines indicate distances between atoms.

Figure 3.

Activity and spectral features of SsTyrH variants. (A) HPLC profiles of SsTyrH variants incubated with Tyr in the presence of H_2O_2 . (B) HPLC profiles of SsTyrH variants reacted with 3-F-Tyr in the presence of H_2O_2 . Negative controls were obtained without enzyme (black). (C) UV-vis absorption spectra of SsTyrH variants. Inset shows the Q band features magnified by 10-fold. (D) EPR spectra of SsTyrH variants.

Figure 4.

Active site views of SsTyrH in complex with 3-F-Tyr. (A) 3-F-Tyr binds in the active site of SsTyrH with two orientations. The gray $F_o - F_c$ omit map is contoured at 3σ . (B) Orientation A has the fluorine substituent facing upward from the heme plane with an occupancy of 0.7. (C) Orientation B has the fluorine substituent facing downward to the heme plane with an occupancy of 0.3. The residue of Leu210 is omitted for clarity. Color code of atoms: carbon, protein, white; carbon, substrate, orientation A, yellow; carbon, substrate, orientation B, cyan; carbon, heme, deep red; fluorine, magenta; nitrogen, blue; oxygen, red. Gray dashed lines indicate distances between atoms.

Figure 5.

Single-crystal UV-vis spectra. (A) Absorption spectra from crystals of 3-F-Tyr-bound complex (black), Tyr-bound complex reacting with H₂O₂ for 20 s (orange), and 3-F-Tyr-bound complex reacting with H₂O₂ for 20 s, consistent with formation of a hydroperoxo intermediate (red). (B) Absorption spectra from crystals of 3-F-Tyr-bound complex (black) and cyanide bound ternary complex (blue).

Figure 6. X-ray crystallographic characterization of a ferric-hydroperoxo intermediate and a cyano ternary complex. (A) Active site views of the ferric-hydroperoxo intermediate with 3-F-Tyr shown in orientation A (0.7 occupancy). (B) Active site views of the ferric-hydroperoxo intermediate with 3-F-Tyr shown in orientation B (0.3 occupancy). (C) Active site views of cyanide bound ternary complex with 3-F-Tyr in overlapped orientations A and B (7:3). Color code of atoms: carbon, protein, white; carbon, substrate, orientation A, yellow; carbon, substrate, orientation B, cyan; carbon, heme, deep red; carbon, cyanide, green; fluorine, magenta; nitrogen, blue; oxygen, red. The gray F_0-F_c omit map is contoured at 3σ . Gray dashed lines indicate distances between atoms.

Figure 7. Possible oxidative pathways for tyrosine activation. (A) Electron transfer (ET) results in a tyrosine cation radical; the aromatic ring faces toward iron with a distance of 5.1 Å. (B) Hydrogen atom transfer (HAT) from the 4-hydroxyl results in a tyrosyl radical; the 4-hydroxyl points away from the iron center, with a distance of 5.9 Å. (C) HAT from one of the two *ortho* carbons results in a tyrosyl radical; the distances from *ortho* carbons to the iron center are 4.9 and 5.7 Å, respectively. R represents the amino acid main chain of Tyr.

Figure 8.

Proposed catalytic mechanisms of TyrH for C-H and C-F bond activation of 3-F-Tyr. The binding orientation of 3-F-Tyr at the enzyme active site dictates C-H (A) and C-F (B) bond cleavage partitioning. R represents the amino and carboxyl groups of 3-F-Tyr.

Table 1.

X-ray Crystallography Data Collection and Refinement Statistics

PDB Code	SeMet SsTyrH	L-Tyr bound binary complex 7KQR	3-F-Tyr bound binary complex 7KQS	CN ⁻ bound ternary complex 7KQT	Ferric-hydroperoxo intermediate 7KQU
Data Collection					
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Cell dimensions					
a, b, c (Å)	47.5, 129.7, 48.4	47.7, 129.8, 48.4	47.5, 129.7, 48.5	47.4, 129.4, 48.4	47.5, 129.3, 48.4
α, β, γ (deg)	90, 94, 90	90, 94, 90	90, 94, 90	90, 94, 90	90, 94, 90
Resolution (Å)	50.00–1.98	50.00–1.89	50.00–1.68	50.00–1.84	50.00–1.58
Redundancy	(2.01–1.98) ^a	(1.92–1.89)	(1.71–1.68)	(1.87–1.84)	(1.61–1.58)
	6.2 (4.5)	3.5 (3.3)	4.7 (3.9)	6.6 (6.4)	4.7 (3.5)
R_{merge}^b (%)	18.1 (72.9)	12.3 (96.2)	11.0 (96.1)	13.4 (94.8)	15.5 (74.9)
$I/\sigma I$	10.8 (1.3)	9.6 (1.2)	17.0 (1.1)	15.2 (1.3)	13.2 (1.1)
Completeness (%)	82.8 (45.0)	97.8 (98.9)	99.8 (98.2)	98.6 (99.4)	99.8 (99.9)
CC _{1/2} , highest resolution shell	0.75	0.57	0.64	0.83	0.63
FOM ^c	0.33				
Refinement					
Resolution (Å)		48.33–1.89	47.42–1.68	48.28–1.84	48.23–1.58
No. of reflections		46,259	66,444	49,387	79,392
$R_{\text{work}}^d/R_{\text{free}}^e$ (%)		16.61/20.73	17.25/20.96	20.29/25.00	15.65/18.87
No. atoms/ B -factors (Å ²)					
Protein		4704/29.01	4744/29.82	4680/42.65	4727/29.22
Heme		86/27.94	86/29.59	86/29.59	86/29.22
Ligand ^f		26/24.24	56/21.86	60/32.83	60/21.33
Solvent		454/36.28	415/38.73	312/49.48	587/42.64
Bond lengths (Å)		0.007	0.006	0.007	0.006
Bond angles (deg)		0.899	0.855	0.883	0.861
Ramachandran analysis ^g					

	SeMet SsTyrH	L-Tyr bound binary complex	3-F-Tyr bound binary complex	CN ⁻ bound ternary complex	Ferric-hydroperoxo intermediate
Favored (%)	96.50	97.68	97.33	98.18	
Allowed (%)	3.00	2.32	2.50	1.82	
Outlier (%)	0.50	0.00	0.17	0.00	

^aNumbers in parentheses refer to data in the highest-resolution shell.

^b $R_{\text{merge}} = \Sigma |h_i| / \Sigma h_i$, where h_i is the observed intensity and $\langle h_i \rangle$ is the average intensity.

^cFOM: figure of merit after PHENIX.Autosol.

^d $R_{\text{work}} = \Sigma ||F_o| - k|F_c|| / \Sigma |F_o|$.

^e R_{free} is the same as R_{obs} for a selected subset (10%) of the reflections that was not included in prior refinement calculations.

^fLigands involved include Tyr, 3-F-Tyr (two binding orientations), cyanide, and hydroperoxo.

^gOutliers in 7KQR are from Gly44, Ser45, and Glu272 from chain A; and the one in 7KQT is Pro110 from chain B. These residues are from the surface flexible region and irrelevant to the active site.

Table 2.Specific Activities of TyrH and Variants at pH 7.0, 55 °C^a

	C–H bond activation of Tyr	C–H bond activation of 3-F-Tyr	C–F bond activation of 3-F-Tyr
SsTyrH	640 ± 20	205 ± 9	102 ± 3
H88A	n.d. ^b	n.d.	n.d.
H88Y	n.d.	n.d.	n.d.
Y230F	580 ± 10	217 ± 5	101 ± 3
Y230H	140 ± 3	117 ± 2	25.4 ± 0.1
H88Y/Y230H	65 ± 9	20.2 ± 0.5	n.d.

^aUnit: nmole·min^{−1}·mg^{−1}.^bn.d.: no detectable activity.

Table 3.
Summary of Heme Ferric Hydroperoxo/Peroxide Intermediates Characterized by X-ray Crystallography

PDB entry	Protein	Resolution (Å)	d of Fe-O (Å)	d of O-O (Å)	θ of Fe-O-O (deg)	Proximal ligand
7KQU	TyrH (this work)	1.6	2.2, 2.4	1.4	128, 138	His
2Z6T	myoglobin	1.2	1.9	1.3	120	His
2VLX	myoglobin	1.3	1.8	1.3	119	His
6L9E	lactoperoxidase	1.7	2.5	1.5	126	His
6LAQ	lactoperoxidase	1.7	2.2	1.6	135	His
5ZWW	lactoperoxidase	1.8	2.2	1.5	133	His
2J5M	chloroperoxidase	1.8	1.9	1.5	131	Cys
4GRC	peroxidase	2.0	2.78	1.5	126	His
1GGF	catalase	2.3	2.6–3.3	1.5	131–135	Tyr