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-F-Tyr) as a substrate. However, little is known about how the enzyme mediates two distinct reactions. Herein, a new TyrH from the thermophilic bacterium Streptomyces sclerotialus (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 mononitrofurfurinated substrate, 3-F-Tyr, which shows one binding conformation but two orientations of the fluorescent atom with a ratio of 7:3, revealing that the primary factor of product distribution is the substrate orientation. During in crystalllo 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 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.

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 Actinomycetes. 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 OrfI3 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 Actinomycetes strains with unknown downstream natural products.6,10 One example is found in the thermophilic bacterium Streptomyces sclerotialus in which a putative TyrH encoded by gene 2337 was proposed through a genome context analysis, 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 peroxygenases. 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, analogs with ring-deactivating substitutions on the 3-position were able to be hydroxylated. The most interesting observation is that LmbB2 could cataclystically 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; 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. However, several metalloenzymes have been acknowledged for their ability to perform defluorination, such as histidine-ligated heme-dependent dehaloperoxidase (DHP) and TyrH, thiolate-ligated heme-dependent cytochrome P450, pterin-dependent nonheme tyrosine/phenylalanine hydroxylase, 2-oxoglutarate-dependent non-heme iron enzymes, Rieske dioxygenase, and thiol dioxygenase. 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.

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. The differences between the halogenated analogs in chemical property, binding affinity, and steric effect could all contribute to the inverted reactivity tendency, but more importantly, these two classes of enzymes use distinct mechanisms. DHP mediates an oxidative C-X bond cleavage and yields quinone products. In contrast, TyrH promotes a nonoxidative C-X bond cleavage and give rise to catechol products.

The ability to perform defluorination is largely associated with the structural features of these biocatalysts; 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, 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.

Here, we isolated the putative TyrH from S. sclerotialus 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 dioxorperoxy intermediate in the pathway of defluorination of 3-F-Tyr.
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. sclerotialus (i.e., SsDDO).\textsuperscript{10} Gene 2337 attracted our attention, as it encodes a putative TyrH from S. sclerotialus (SsTyrH). This putative TyrH shares 43% protein sequence identity with LmbB2 as indicated by pairwise sequence alignment through EMBoss Needle.\textsuperscript{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.\textsuperscript{24,25} Upon the addition of Tyr (1 mM), the Soret band decreased with a 1.5 nm redshift of the λmax 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 (red trace). The heme cofactor in SsTyrH as indicated by a slightly sharper high-spin signal with \( g_1 = 5.82, g_∥ = 2.00 \) (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 \( g_1 = 5.82, g_∥ = 2.00 \) (blue trace, Figure 1D). Similar UV-visible 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 \( \mu \)M. The binding stoichiometry was
in the range of 0.7–0.8 for both substrates, as indicated by the breakpo{
}ints. 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. 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,1 upon reacting with $\text{H}_2\text{O}_2$, 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 1E). 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 deflourination 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 activity at pH 7.0 and 50 °C (Figure S3). Steady-state kinetic assay of SsTyrH with Tyr gave a $K_M$ of 530 ± 50 μM and a $k_{cat}$ of 14.2 ± 0.5 min⁻¹ at room temperature. In the case of 3-F-Tyr, the values are 740 ± 90 μM and 4.7 ± 0.2 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 ± 0.1 min⁻¹, a $K_M$ of 940 ± 50 μM, 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 simulta-

eously; 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 $\text{H}_2\text{O}_2$-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 $P_2_1$ 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 Cα atoms. The buried surface area between two subunits is 1427 Å², corresponding to 10% of the total

**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 α carbon, protein, white; carbon, substrate, yellow; carbon, heme, deep red; nitrogen, blue; oxygen, red. The $F_o−F_c$ omit maps are contoured at 3 $σ$, colored in gray and blue for heme and Tyr, respectively. Gray dashed lines indicate distances between atoms.
Hisⁿ from α thus, isolated from bulk solvent. As shown in Figure 2C, the protein surface as indicated by its apparent electron density. The distal pocket of the heme center is shielded and, the heme-binding pocket surrounded by short helices (α1t o α11), two short β-strands (β1 and β2), and four 3₁₀ helices (η1 to η4) (Figure 2B). Along with a short two-stranded β-sheet, a helical bundle formed by three long α-helices (α2, α3, and α7) separate the molecule from the center, flanked by other α-helices and 3₁₀ helices. Two α-helices (α1 and α10) and a bent α-helix (α11) locate behind the long helical bundle, and a heme-binding pocket surrounded by short helices (η1, η2, α4, α5, α8, and α9) 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 α7 is the proximal ligand of the heme and it coordinates to the iron with the Nζ. 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. 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 ± 10 nmol-min⁻¹-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 H88 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 product, indicating that His88 is critical for catalysis but not Tyr230.

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

<table>
<thead>
<tr>
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<th>C–H bond activation of 3-F-Tyr</th>
<th>C–F bond activation of 3-F-Tyr</th>
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<tbody>
<tr>
<td>SsTyrH</td>
<td>640 ± 20</td>
<td>205 ± 9</td>
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<tr>
<td>H88Y</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Y230F</td>
<td>580 ± 10</td>
<td>217 ± 5</td>
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<tr>
<td>Y230H</td>
<td>140 ± 3</td>
<td>117 ± 2</td>
</tr>
<tr>
<td>H88Y/Y230H</td>
<td>65 ± 9</td>
<td>20.2 ± 0.5</td>
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“Unit: nmole-min⁻¹-mg⁻¹. n.d.: no detectable activity.

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 λmax 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 the 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 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 Kd of 109 ± 5 μM. 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 Cα hydrogen peroxide-containing mother liquor for various time crystals of the 3-F-Tyr-bound SsTyrH complex were soaked in main chain carbonyl of Ser157. The structural determination of interacts with His88, while in orientation B it interacts with the substrate, Tyr. In orientation A, the fluorine substituent facing upward (orientation A, Figure 4B) and downward (orientation B, Figure 4C) to the heme porphyrin plane, respectively. 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 F0−F− 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 both 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-hydroxylation 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. 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 small differences of λmax between crystalline and solution states are anticipated, resulting from crystal packing and pH difference as reported in a nonheme dioxygenase study. In addition, the absorbance of Soret peaks was easily saturated due to the dense packing of crystals, hence the readings of λmax based on the peak shape could introduce some inaccuracy. After reacting with H2O2, 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. 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; such a ferric-hydroperoxo intermediate was also reported in histidine-ligated heme oxygenase with absorption spectra maxima at 421, 530, and 557 nm. 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 H2O2 for 20 s yielded an intermediate corresponding to a ferric-hydroperoxo, also known as compound 0 (Cpd 0, Fe3+-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 Cα 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 pKₐ 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. 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 cyanide ternary complex structure with the ferric-hydroperoxo intermediate. The Soret band of the cyanide-soaked crystal redshifted to 412 nm, and the spectral features in the α/β region became unresolvable with features centered at 535 and 560 nm (Figure S1B). 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 Ca 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 peroxo bond 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. 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 pKₐ of the corresponding compound II (Cpd II, a ferryl-oxo with a neutral porphyrin). A known example is DHP, which utilizes ET to activate aromatic substrates through peroxynase-like or peroxidase-based mechanism. 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. 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. Substrate analogs including phenylalanine and O-methyl-tyrosine bind the enzyme, but with no reactivity. 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. 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. 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\textsubscript{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,54,56 horseradish peroxidase,55 heme oxygenase,57 etc. In this study, we took advantage of the significantly altered kinetics in cr\textit{istallo} 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 H\textsubscript{2}O\textsubscript{2} concentration, demonstrating that the reactions are independent (not processive) and that a single pathway could not yield both products.\textsuperscript{3} The C–H bond and C–F bond cleavage reactions also differ by two electrons (F\textsuperscript{−} vs H\textsuperscript{+} 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 H\textsubscript{2}O\textsubscript{2} to O\textsubscript{2}.\textsuperscript{3} In

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

<table>
<thead>
<tr>
<th>PDB entry</th>
<th>Protein</th>
<th>Resolution (Å)</th>
<th>d of Fe–O (Å)</th>
<th>d of O–O (Å)</th>
<th>θ of Fe–O–O (deg)</th>
<th>Proximal ligand</th>
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<tr>
<td>7KQU</td>
<td>TyrH</td>
<td>1.6</td>
<td>2.2, 2.4</td>
<td>1.4</td>
<td>128, 138</td>
<td>His</td>
</tr>
<tr>
<td>2Z6T</td>
<td>myoglobin</td>
<td>1.2</td>
<td>1.9</td>
<td>1.3</td>
<td>120</td>
<td>His</td>
</tr>
<tr>
<td>2VLX</td>
<td>myoglobin</td>
<td>1.3</td>
<td>1.8</td>
<td>1.3</td>
<td>126</td>
<td>His</td>
</tr>
<tr>
<td>6L9E</td>
<td>lactoperoxidase</td>
<td>1.7</td>
<td>2.5</td>
<td>1.5</td>
<td>135</td>
<td>His</td>
</tr>
<tr>
<td>6LAQ</td>
<td>lactoperoxidase</td>
<td>1.7</td>
<td>2.2</td>
<td>1.6</td>
<td>135</td>
<td>His</td>
</tr>
<tr>
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<td>lactoperoxidase</td>
<td>1.8</td>
<td>2.2</td>
<td>1.5</td>
<td>133</td>
<td>His</td>
</tr>
<tr>
<td>2JSM</td>
<td>chloroperoxidase</td>
<td>1.8</td>
<td>1.9</td>
<td>1.5</td>
<td>131</td>
<td>His</td>
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<td>2.6–3.3</td>
<td>1.5</td>
<td>131–135</td>
<td>Tyr</td>
</tr>
</tbody>
</table>
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-F-Tyr to bind before H$_2$O$_2$ in order to proceed with C–F bond cleavage proceeding with a distinct mechanism. The Menten type kinetics, while C–F bond activation may not be necessary for the native reaction.

In the ferric-hydroperoxo structure, the distal oxygen of the peroxo is 3.9 and 4.6Å 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.\textsuperscript{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.

### Conclusion

Herein, the TyrH from \textit{S. sclerotiorum} 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,\textsuperscript{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 \textit{S. sclerotiorum} was cloned into the pET28a-TEV expression vector (GenScript). The plasmid of N-terminally His$_6$-tagged SsTyrH was then transformed into the \textit{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.\textsuperscript{10} The protein purification was conducted following the previously reported procedure.\textsuperscript{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 His$_6$-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 Ke measurements of Tyr and 3-F-Tyr using UV–vis spectroscopic titration were recorded in Supporting Information.

EPR spectra 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 E500 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.\textsuperscript{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, at room temperature in 100 mM potassium phosphate with 50 mM NaCl and pH 8.0. SsTyrH or variants (100 μM) 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 μL of concentrated HCl (6 M) was added to quench the reaction. The final volume was 200 μL. 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-μL portion of filtrate was injected into an InertSustain C18 column (5 μm particle size, 4.6 × 100 mm, GL Sciences Inc.) with a flow rate of 1 mL/min, and then analyzed by a Thermo Scientific Ultimate-3000RSD 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 cocrystallization with a substrate, either Tyr or 3-F-Tyr. His-tagged concentrated protein was supplemented with either Tyr (2 mM) or 3-F-Tyr (3 mM). The untagged SsTyrH was prepared in 50 mM Superdex-75 column (GE Healthcare) was used to further purify the protein. The untagged protein was injected into an InertSustain C18 column (5 μm particle size, 4.6 × 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.

**Preparation of the Unreactive/Reactive Termary 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 (5 mM) 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.

**REFERENCES**

Supporting Information

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

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**Ligand binding as monitored by UV-vis spectroscopic titration**

Titration experiments were performed by measuring UV-vis spectra of the enzyme alone and after each sequential addition of ligand. Spectra were corrected for dilution and baseline adjusted. A typical experiment used 9~10 μM of TyrH in 50 mM Tris-HCl with 50 mM NaCl at pH 8.0, and the ligand was titrated from 0.7 to 94.1, 123.5, or 444 μM for wild-type, Y230F, or Y230H, respectively. Spectra were acquired with a Lambda 25 UV-vis spectrophotometer (PerkinElmer). Stock solutions of ligand (Tyr or 3-F-Y) were dissolved in double-deionized H$_2$O. Data were analyzed with linear regression in the case of tight binding or fitted to a hyperbola (eq. 1) to determine the dissociation constant for weak binding.

$$\Delta \text{Absorbance} = \frac{\Delta \text{Abs}_{\text{max}} \times [L]}{(K_D + [L])} \quad (1)$$

**The optimal pH and temperature investigation on SsTyrH-mediated reaction**

Enzymatic reactions were set up by adding 1 mM H$_2$O$_2$ to 10 μM enzyme pre-incubated with 1 mM substrate to a final volume of 200 μL. 10 μL of 12 M HCl was added to quench the reaction after mixing at 900 rpm for 1 min (Thermomixer R, Eppendorf). To determine the optimal pH, reactions were set up at room temperature in various buffers: 50 mM MES/NaOH at pH 6.0, 50 mM MOPS/NaOH at pH 7.0, 50 mM Tris/HCl at pH 8.0, 50 mM CHES/NaOH at pH 9.0, and 50 mM CAPS at pH 10.0. All buffers were adjusted by NaCl to a final ionic strength of 150 mM. Buffers outside this pH range (pH 6 ~ 10) denatured the enzyme.

To determine the optimal temperature, reactions were set up in 50 mM sodium phosphate buffers at various temperatures. Sodium phosphate buffers were adjusted to different pH values at room temperature with the consideration of temperature coefficient to ensure that the buffers were at pH 7.0 at the reaction temperatures. All buffers were adjusted with NaCl to reach a final ionic strength of 150 mM. A Thermo Scientific Ultimate-3000SD HPLC rapid separation system with an Inertil column (ODS-3 3 μm, 4.6 × 100 mm, GL Sciences Inc.) was used to separate the reaction mixture. The same column was used to generate the data presented in SI. Additional sample handling and HPLC analyses followed the method described in the main text. HPLC standard curve of DOPA was used to quantitate the product formation in each reaction. Errors were standard deviations by conducting the same reaction in triplicate.

**Steady-state kinetics of SsTyrH**

Kinetic assays were set up by adding 1 mM H$_2$O$_2$ to 10 μM enzyme pre-incubated with substrate at various concentrations to a final volume of 500 μL in 50 mM sodium phosphate at pH 7.0 with NaCl to reach an ionic strength of 150 mM. The reactions were mixed at 900 rpm for 1 min (Thermomixer R, Eppendorf), and 100 μL aliquots were quenched with 5 μL, 12 M HCl. Additional sample handling and HPLC analyses followed the method described above and in main text. Products were quantitated using HPLC standard curves. 3-F-5-OH-Tyr is not commercially available, so a standard curve was generated by measuring the depletion of 3-F-Tyr using the H88Y/Y230H variant which only yields 3-F-5-OH-Tyr. Initial rates at different substrate concentrations were obtained by linear fitting. Errors were standard deviations by conducting the same reaction in triplicate. Initial rates were plotted versus substrate concentration and fit with the Michaelis–Menten equation (eq. 2) or the Hill equation (eq. 3) as appropriate.

DOI: 10.1021/jacs.1c00175 (SI)
\[
\frac{v_0}{[E]} = \frac{k_{cat} \times [S]}{(K_M + [S])}
\] (2)

\[
V = \frac{V_{\text{max}} \times [S]^n}{(K^n + [S]^n)}
\] (3)

The oxygen-dependent activity of SsTyrH

The assay method of oxygen-dependent activity was derived from a previous Orf13 study.\(^3\) SsTyrH (100 \(\mu\)M) was incubated with either Tyr or 3-F-Tyr (3 mM) for 1 h, in the presence of ascorbic acid (25 mM). Additional sample handling and analyses followed the method described above and in the main text.

The specific activity of SsTyrH and variants

To measure the specific activity of SsTyrH and variants, enzymatic reactions were set up by adding 1 mM \(\text{H}_2\text{O}_2\) to 10 \(\mu\)M enzyme pre-incubated with 1 mM substrate to a final volume of 200 \(\mu\)L. 10 \(\mu\)L of 12 M H\(_2\)Cl was added to quench the reaction after mixing at 900 rpm for 1 min in buffer (50 mM sodium phosphate at pH 7, adjusted to 150 mM ionic strength with NaCl) at 50 °C. Additional sample handling and analyses followed the method described above and in main text. Products were quantitated as in the steady-state kinetics above. Errors were standard deviations by conducting reactions in triplicate.

X-ray diffraction data collection and structure determination

All structural data were collected at 100 K and processed using HKL2000.\(^2\) Single-wavelength anomalous diffraction data for the SeMet-substituted TyrH were collected from beamline SSRL 9-2, and a 1.98-Å resolution dataset was obtained at a wavelength of 0.97903 Å, corresponding to an absorption peak of selenium. PHENIX.AutoSol\(^3\) and PHENIX.AutoBuild\(^4\) were used for phasing and model building. A structure of SsTyrH in complex with \(\alpha\)-tyrosine (Tyr) was determined from the data collected at beamline SSRL 9-2 with a resolution of 1.89 Å, which was later termed an initial model for molecular replacement and refinement for other structures using PHENIX\(^5\) software packages.

DNA sequence and mutagenesis primers of SsTyrH

The codon-optimized DNA sequence of SsTyrH was deposited to GenBank, with accession number MW418022. Mutagenic primers were synthesized by Integrated DNA Technologies. Mutagenesis primers for H88A, H88Y, Y230F, and Y230H were based on the codon-optimized DNA sequence. The sequences of the forward primers are listed below, and its reverse pairs are the reverse complement of the forward primers. The mutation sites are underlined.

5′– G\(_{10}\)GGATCACGGGC\(_{10}\)GGTTACCTTTATTC–3′ for H88A
5′– G\(_{10}\)GGATCACGGG\(_{10}\)GGTTACCTTTATTC–3′ for H88Y
5′– G\(_{10}\)TGGCTGGGTGGT\(_{10}\)TCTGATAACTATTTTCTGAC–3′ for Y230F
5′– G\(_{10}\)TGGCTGGGTGGT\(_{10}\)TCTGATAACTATTTTCTGAC–3′ for Y230H
Table S1. UV-vis and EPR spectral features of SsTyrH aqueous or frozen solution samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>UV-vis spectral features (nm)</th>
<th>EPR spectral features (g value)</th>
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<tr>
<td></td>
<td>Soret band</td>
<td>Q/CT bands</td>
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<tr>
<td>Wild-type (WT)</td>
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</tr>
<tr>
<td>WT + 1 mM Tyr</td>
<td>405</td>
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<td>WT + 1 mM 3-F-Tyr</td>
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<tr>
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</tr>
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<td>H88Y</td>
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Table S2. Distances (d) and angles (θ) between the heme iron and axial ligands

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<th>Structure</th>
<th>Parameter</th>
<th>Subunit A</th>
<th>Subunit B</th>
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<tr>
<td>Tyr bound binary complex (7KQR)</td>
<td>d of Fe-N$_{\text{His196}}$</td>
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<td>2.4 Å</td>
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<td>3-F-Tyr bound binary complex (7KQS)</td>
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<td>2.5 Å</td>
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<tr>
<td>Cyanide bound ternary complex (7KQT)</td>
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<td>d of Fe-C</td>
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<td>1.9 Å</td>
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<td>d of C-N</td>
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<td>1.2 Å</td>
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<tr>
<td></td>
<td>θ of Fe-C-N</td>
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<td>167°</td>
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<tr>
<td>Ferric hydroperoxide intermediate (7KQU)</td>
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<td>θ of Fe-O$_1$-O$_2$</td>
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<td>128°</td>
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**Fig. S1. Size-exclusion chromatography.** Black trace: gel filtration markers purchased from Sigma-Aldrich. Peak 1: alcohol dehydrogenase, 150 kDa MW, eluted at 48 mL; peak 2: albumin, 66 kDa MW, eluted at 52 mL; and peak 3: carbonic anhydrase, 29 kDa MW, eluted at 67 mL. Red trace: Purified, untagged SsTyrH. The major fraction eluted at 58 mL, which is consistent with a monomer of 34 kDa MW of SsTyrH calculated based on the protein sequence. Size exclusion column and elution buffer: 16/600 Superdex 75 pg with 50 mM Tris-HCl and 50 mM NaCl at pH 8.
Fig. S2. UV-vis spectroscopic titration studies of SsTyrH and variants with Tyr or 3-F-Tyr
(A) SsTyrH with the addition of Tyr. Baseline subtracted spectra (left), difference spectra (middle), and the difference in absorbance (peak minus trough) as a function of the ratio of ligand to TyrH (right). (B) SsTyrH with the addition of 3-F-Tyr. Baseline subtracted spectra (left), difference spectra (middle), and the difference in absorbance (peak minus trough) as a function of the ratio of ligand to TyrH (right). (C) Y230F variant with addition of Tyr. Baseline subtracted spectra (left), difference spectra (middle), and the difference in absorbance (peak minus trough) as a function of the ratio of ligand to TyrH (right). (D) Y230H variant with addition of Tyr. Baseline subtracted spectra (left), difference spectra (middle), and the difference in absorbance (peak minus trough) as a function of ligand concentration (right). Linear fitting shows tight binding of the ligand, or hyperbolic fitting (eq. 1) is used to determine $K_D$. Spectra of the resting enzyme are shown in black, the endpoint in dark blue, and intermediate ligand concentrations in light blue.
Fig. S3. The optimal pH and temperature of SsTyrH-mediated hydroxylation
(A) Specific activities of SsTyrH with Tyr at room temperature in buffers between pH 6 to 10.
(B) Specific activities of SsTyrH with Tyr at various temperatures in buffers of pH 7.0.
Fig. S4. Steady-state kinetic assays of SsTyrH with Tyr and 3-F-Tyr at pH 7.0, room temperature. (A) Reaction with Tyr. (B) C-H bond and (C) C-F bond cleavage reactions with 3-F-Tyr. The fittings (eq. 2 for A and B, eq. 3 for C) are shown with red lines.
Fig. S5. HPLC profiles of oxygen-dependent reactions catalyzed by SsTyrH
SsTyrH generated minimal product(s) when reacted with Tyr (A) or 3-F-Tyr (B), in the presence of ascorbate under aerobic conditions. Chromatograms of reactions are shown in black, and negative controls without enzyme are shown in gray.
Fig. S6. Protein sequence alignment of TyrHs from *Streptomyces* strains. Strictly conserved residues are colored in white with a red background. The secondary structure elements are defined by SsTyrH. The axial histidine ligand for heme is highlighted by a round mark; rhombus and triangles highlight residues involved in the binding of heme and substrate, respectively. The included sequences are SsTyrH from *S. sclerotialus* (WP_051872337), LmbB2 from *S. lincolnensis* (AXG51384), Por14 from *S. albus* (AEA29637), Orf13 from *S. refuineus* (ABW71844), SibU from *Streptosporangium sibiricum* (ACN39744), HrmE from *S. griseoflavus* (AEH41783), and Tomi from *S. achromogenes* (ACN39022).
Fig. S7. UV-vis and EPR spectra of SsTyrH variants. Spectra of enzyme alone and ES complex are colored in black and red, respectively. Variants are (A) H88A, (B) H88Y, (C) Y230F, (D) Y230H, and (E) H88Y/Y230H. EPR spectra were collected at 10 K, with microwave power of 1.0 mW.

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**Fig. S8. The residue environment of His88.** His88 is stabilized by the backbone carbonyl group of Trp84, and forms π-π stack with Phe234. Distances (Å) are highlighted by orange dashed lines.
Fig. S9. Refinement results of SsTyrH and 3-F-Tyr binary complex dataset using alternate models. (A) Refinement with L-tyrosine (Tyr) resulted in positive electron density maps on both ortho carbons. (B) Refinement with orientation A. Negative and positive electron density maps were observed on C3 and C5 carbons, respectively. (C) Refinement with orientation B. Negative and positive electron density maps were observed on C3 and C5 carbons, respectively. (D) Refinement with orientations A and B and a relative ratio of 7:3. No residual electron density map was observed. The light blue $2F_o - F_c$ electron density maps are contoured at 1 σ. The $F_o - F_c$ density maps contoured at 3.0 σ and -3.0 σ are shown in green and red, respectively.
Fig. S10. Absorption spectra of oxidized and reduced SsTyrH in complex with 3-F-Tyr
(A) Ferric enzyme in complex with 3-F-Tyr before (black) and after (red) anaerobic incubation with 1 mM dithionite in the solution state. The chemically reduced sample showed spectral features at 427, 556, and 581 nm. (B) Ferric enzyme in complex with 3-F-Tyr before (black) and after (red) anaerobic incubation with 5 mM dithionite in crystalline state. The chemically reduced crystal showed spectral features at 430, 554, and 580 nm. The UV-vis spectra of single crystals included in this figure were recorded by a QDI 2010 microspectrophotometer (CRAIC Technologies).
Fig. S11. Refinement results on Cpd 0 intermediate with different models

(A) Refinement with one oxygen atom, resulting in positive electron density. (B) Refinement with Cpd 0 at full occupancy, resulting in negative electron density. (C) Refinement with Cpd 0 at 0.8 occupancies, resulting in no residual electron density. The light blue $2F_o - F_c$ electron density maps are contoured at 1 $\sigma$. The $F_o - F_c$ density maps contoured at 3.0 $\sigma$ and -3.0 $\sigma$ are shown in green and red, respectively.
Fig. S12. UV-vis spectra of cyanide bound complexes of SsTyrH in solution
The spectra are the ligand-free SsTyrH (black), bound with cyanide (green), bound with cyanide and Tyr (blue), and bound with cyanide and 3-F-Tyr (red), respectively.
Fig. S13. Side views of ferric-bound hydroperoxo intermediate of TyrH in complex with 3-F-TyrH. In both orientations, the distances from distal oxygen of hydroperoxo to two ortho carbon atoms are 3.9 and 4.6 Å.
Fig. S14. Structural superposition of SsTyrH with SfmD. SsTyrH (PDB entry: 7KQR) is shown in gray color in the cartoon with heme group in red. SfmD (PDB entry: 6VDQ) is shown in orange color with heme group in orange. The cartoon of SfmD is colored from light to dark, representing the transition from N- to C-terminus.
References