Substrate-Assisted Hydroxylation and O-Demethylation in the Peroxidase-like Cytochrome P450 Enzyme CYP121

Romie C. Nguyen, Yu Yang, Yifan Wang, Ian Davis, and Aimin Liu*

ABSTRACT: CYP121 is a P450 enzyme from Mycobacterium tuberculosis that catalyzes a C–C coupling reaction between the two aromatic rings on its native substrate cyclo(L-Tyr-L-Tyr) (cYY) to form mycocyclosin, a necessary product for cell survival. Unlike the typical P450 enzymes for hydroxylation, CYP121 is believed to behave like a peroxidase and conduct radical-mediated C–C bond formation. Here, we probe whether the phenolic hydrogen of the substrate is the site of the postulated hydrogen atom abstraction for radical formation. We synthesized a singly O-methylated substrate analogue, cYF-4-OMe, and characterized its interaction with CYP121 by ultraviolet-visible and electron paramagnetic resonance spectroscopies and X-ray crystallography. We found that cYF-4-OMe can function as a substrate of CYP121 using the established assay via the peroxide shunt. Analysis of the enzymatic reaction revealed an O-demethylation of cYF-4-OMe instead of cyclization, yielding cYY and formaldehyde. A hydroxylated substrate, cYF-4-OMeOH, is expected to be the intermediate product, which was trapped and structurally characterized by X-ray crystallography. We further determined that the deformylation reaction of cYF-4-OMeOH proceeds via an alkyl–oxygen rather than aryl–oxygen bond cleavage by the 18O-labeling study also invokes the diradical mechanism.11 Although the computational study also invokes the diradical mechanism.11 Although the substrate-based radical intermediate remains to be trapped and characterized, its participation in the catalytic cycle is analogous to C–C bond formation from two aromatic residues in the biogenesis of the tryptophan tryptophylquinone cofactor in methyamine dehydrogenase.12 The diradical mechanism is also supported by experimental evidence on alternate substrates. CYP121 is found to possess peroxidase-like catalytic activity capable of oxidizing peroxide substrates to their radical forms and is therefore labeled as P450 peroxidase.10 The catalytic center of CYP121 is a heme-thiolate, which is similar to other P450 enzymes. The co-crystallized CYP121-cYY complex shows that cYY binds with its phenol groups at nearly 90° to each other. This binding of cYY minimally alters...
the structure of the enzyme and causes nearly non-observable changes in the active site architecture. In the first step of a general P450 mechanism, substrate binding results in loss of the axial water ligand, leading to an open coordination site to the heme iron, converting it from a six-coordinate low-spin ferric state to a five-coordinate high-spin species for further reduction and O2 binding. However, this is not the case for CYP121. The CYP121-cYY complex remains mostly at the low-spin ferric state, and the high-spin species is minimal as measured by electron paramagnetic resonance (EPR) and ultraviolet–visible (UV−vis) spectroscopies. To understand these observations that are contrary to how P450 enzymes generally behave upon substrate binding, an electron-nuclear double resonance (ENDOR) spectroscopic study of CYP121 has been described in our previous study. The solvent-derived distal ligand at the ferric heme iron is shown to be in dynamic equilibrium among water, hydroxide, and the ligand-free form, which is affected by temperature and pH. The binding of cYY to the distal pocket of the heme alters the equilibrium and weakens the solvent-derived ligand affinity to the iron ion but does not cause a purely ligand-free heme center, yet the heme has a 100-fold higher affinity to cyanide than the enzyme alone without cYY.

Figure 1. Binding characteristics of cYY and cYF−4-OMe with CYP121 as measured by UV−vis and EPR spectroscopies. CYP121 bound with no added ligand (black), cYY (blue), and cYF−4-OMe (red) was measured by (A) UV−vis spectroscopy of ferric form of CYP121 (5 μM) with cYY (300 μM) or cYF−4-OMe (100 μM), (B) EPR spectroscopy of CYP121 (200 μM) at 10 K and 1 mW with cYY (400 μM) or cYF−4-OMe (200 μM), and (C) g = 2 region EPR spectra at 50 K and 0.2 mW. A spectral shift is observed upon ligand binding. Note that full binding is not achieved for cYF-4-OMe due to the low solubility in buffer, which presents itself as a shoulder that is similar to unbound enzyme.

In the present work, we aim to probe the first catalytic step of the CYP121 pathway after the formation of the enzyme-based oxidant. A single tyrosine moiety of the substrate was modified from a phenol to an aryl methoxy group to probe the ability of the enzyme to handle the lack of an easily removable hydrogen atom. The investigation of the synthetic probe with

Scheme 1. Reaction of CYP121 with the Native Substrate

(A) cYY to form mycocyclosin via a C−C bond coupling cyclization reaction and (B) designed synthetic probe cYF−4-OMe to form cYY as a product.

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the CYP121-mediated reaction not only confirmed the catalytic importance of the hydroxyl group of cYY during the initial step of catalytic oxidation mediated by a heme-based oxidant but also yielded a surprising outcome that elucidates the intrinsic P450 catalytic characteristics of the heme center in this peroxidase-like P450 enzyme.

■ RESULTS

Enzyme-Substrate Interaction with the Synthetic Probe Cyclo(\textit{L}-Tyr-\textit{L}-Phe-4-OMe). Cyclo(\textit{L}-Tyr-\textit{L}-Phe-4-OMe) (cYF-4-OMe) is a cyclodipeptide in the \textit{l}-configuration with a single tyrosine moiety modified to phenylalanine with a methoxy group located in the fourth position on the phenyl ring. This modification was chosen to interrogate the importance and role of the hydroxyl group in the catalytic cycle of CYP121. The compound was synthesized using previously published methods on chiral piperazine synthesis with minor modifications (Supporting Information, Scheme S1).\textsuperscript{10,16,17} The characterization of this synthetic probe by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy is described in Figure S1.

The binding of cYF-4-OMe to CYP121 shares similar spectroscopic characteristics with cYY in the electronic absorption spectra (Figure 1A). In the absence of a ligand, CYP121 exhibits an expected Soret band centered at 416 nm with a shoulder at 358 nm as well as \(\alpha/\beta\) bands at 539 and 566 nm.\textsuperscript{10} Upon addition of cYF-4-OMe, the Soret band blue shifts from 416 to 394 nm, a more pronounced version of the shift caused by cYY binding, which is indicative of type 1 binding where a water ligand is displaced from the heme iron. Differences are also observed in the \(\alpha/\beta\) region for both cYY and cYF-4-OMe binding, where the 538 and 565 nm peaks become broadened. The broad feature in the cYF-4-OMe-enzyme complex is similar in shape to the feature seen in cYY binding, yet it is slightly blue shifted with an observable increase in signal intensity. The charge transfer band at 645 nm is observed in the spectra of both compounds, although the cYF-4-OMe band has higher absorbance than with cYY. Overall, cYF-4-OMe binding causes more observable changes of the electronic structure of the heme center compared to changes caused by cYY binding.

EPR spectroscopic characterization revealed a more pronounced spectral change upon cYF-4-OMe binding as compared to cYY. In the EPR spectrum of cYF-4-OMe bound to CYP121, a large high-spin ferric signal \((g_{\alpha} = 1.69; g_{\sigma} = 3.55;\) and \(g_{\beta} = 8.04)\), which is absent from free enzyme and minor in the CYP121-cYY complex, is observed coupled with a decrease in the low-spin signal of the ferric heme in the resting state (Figure 1B). The low- to high-spin conversion, which is missing from the binding of the native substrate cYY,\textsuperscript{10,15} indicates either the loss of a solvent-derived axial ligand from the ferric heme or substantially weakened binding to the heme upon binding of the synthetic probe. However, such a new high-spin ferric heme EPR signal of CYP121 is commonly seen in many other P450 enzyme-substrate complexes with typical hydroxylation catalytic activities.\textsuperscript{18,19}

The minor rhombic, low-spin ferric heme species of the enzyme-probe complex were measured at nonsaturating conditions for the low-spin species (50 K and 0.20 mW) to ensure accurate line shapes and intensities for comparison (Figure 1C). The low-spin ferric heme signal in the cYY-bound enzyme \((g_{\sigma} = 1.90; g_{\alpha} = 2.25;\) and \(g_{\beta} = 2.46)\) and ligand-free \((g_{\sigma} = 1.88; g_{\alpha} = 2.25;\) and \(g_{\beta} = 2.50)\) presents distinct g values. The enzyme-substrate complex of the low-spin EPR signal also has a slightly decreased rhombicity, that is, \(\Delta g = (g_{\sigma} − g_{\alpha})\). These results suggest that the synthetic probe cYF-4-OMe binds to the heme distal pocket and causes the departure of the solvent-derived ligand as the major conformation and a minor fraction retains the solvent-derived ligand. The EPR spectra showed a near but not full conversion to the bound state upon addition of cYF-4-OMe (Figure 1C).

A crystal structure of CYP121 in complex with cYF-4-OMe (Figure 2) was obtained through cocrystallization and refined to 1.39 Å resolution (Table 1). At pH 7.4, the crystal structure shows that cYF-4-OMe binds exclusively with the methyl group pointing toward the heme. This is consistent with all but one, that is, cyclo(\textit{l}-Tyr-\textit{l}-DOPA), of the known substrate analogues thus far reflecting the scaffold of cYY bound in the distal pocket of CYP121,\textsuperscript{21} suggesting a specific protein–cYY interaction at the remote tyrosine moiety and a relatively relaxed environment near the heme center for the primary substrate and external oxidant. Interestingly, the presence of the methyl group on cYF-4-OMe pointing toward the heme does not show any electron density above the heme.

![Figure 2. Cocrystralized structure of CYP121 in complex with cYF-4-OMe at 1.39 Å resolution. The \(F_{o} − F_{c}\) density map (green) was contoured to 3 \(\sigma\) and the density was fitted with the ligand structure (yellow). A single binding orientation of the synthetic probe was observed in the active site with the methoxy group pointing to the heme.](https://dx.doi.org/10.1021/acscatal.0b04596)
iron, where a water ligand was seen in the substrate-free structure. The lack of this additional small electron density is contrary to the EPR data presented above and is likely due to low occupancy. The EPR data shows that a very minor fraction of the probe-bound CYP121 remains to be associated with a solvent-derived ligand at the distal side of the heme, rendering it unobservable in X-ray crystallography.

Together with the previous EPR/ENDOR study, the solvent-derived ligand should weaken its interaction with the heme iron in this minor fraction of the complex. The high-resolution crystal structure of the enzyme-probe complex is thus in agreement with the EPR characterization of the heme center.

**Synthetic Probe as an Alternate Substrate.** The catalytic activity of CYP121 with cYF-4-OMe was probed using the previously established peroxide shunt pathway using H$_2$O$_2$ or peracetic acid as the oxidant. This reaction resulted in the appearance of a single detectable new peak in the high-performance liquid chromatography (HPLC), which eluted with a retention time of 6.3 min compared to the retention time of cYF-4-OMe at 8.3 min (Figure 3A). This new eluate has the same retention time with cYY on the column under the same conditions, and it has an [MH]$^+$ ion m/z 327, the same mass that has been reported previously for cYY (Figure S1B). The new eluate at 6.3 min was also analyzed by UV–vis, and the absorbance spectra matches the known optical spectra of cYY (Figure 3B). These results point toward loss of a methyl group from cYF-4-OMe and formation of cYY as the product of the CYP121-mediated reaction, suggesting an oxidative C–O bond cleavage event that is rarely observed for a P450 enzyme.

**Identification of the Secondary Product of the CYP121 Reaction with cYF-4-OMe.** Depending on the mechanism for cYY formation from cYF-4-OMe, methanol or formaldehyde may be formed. If CYP121 follows a peroxidase-like reaction with the alternate substrate, methanol would be formed. Conversely, formaldehyde can be formed if the enzyme follows a classic P450-like reaction with the alternate substrate, methanol or formaldehyde dehydrogenase (ADH) and formaldehyde dehydrogenase (FDH), which can

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**Table 1. Data Collection and Refinement Statistics for cYF-4-OMe Crystal Structure and Its Hydroxylated Intermediate cYF-4-OMeOH**

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Values in parentheses are for the highest resolution shell. $^b$Rmerge = $\sum_h \sum_l |I_h(lk\ell) - \langle I_h(lk\ell)\rangle/\sum_h \sum_l I_h(lk\ell)$, where the sum is over all the i measured reflections with equivalent Miller indices hkl, $\langle I_h(lk\ell)\rangle$ is the averaged intensity of these i reflections, and the grand sum is over all measured reflections in the dataset. $^c$According to Karplus and Diederichs. $^d$All positive reflections were used in the refinement. $^e$According to Engh and Huber. $^f$Calculated by using MolProbity.
be used to detect the presence of methanol or formaldehyde, depending on whether NAD⁺ or NADH is used as the cofactor.24,25 The ADH reaction is bidirectional, depending the availability of the cofactor, whereas the FDH reaction is unidirectional. After the CYP121 reaction was completed with cYF-4-OMe and peracetic acid, CYP121 was removed with a 10 kDa cut-off membrane filter in a concentrator, and ADH or FDH (10 μM) and NAD⁺ or NADH (200 μM) were mixed with the flow-through, and the absorbance was monitored at 339 nm for NADH formation or loss, respectively.

All attempts to detect and quantify methanol as a side reaction product were unsuccessful. No notable formation of NADH was observed from the reaction mixture containing ADH and NAD⁺ (Figure 4A). However, when testing for the presence of formaldehyde by running the ADH reaction in reverse, a decrease in NADH absorbance was observed (Figure 4B). As illustrated in Scheme 1B, these assay results support the formation of formaldehyde as the secondary product of the CYP121-mediated cYF-4-OMe oxidation reaction. The values of the initial and final absorbance were converted to concentration of NADH using an NADH standard curve (Figure S2A) and then subtracted to yield the concentration of formaldehyde. The FDH control showed 21.4 ± 3.6 μM loss of NADH via light-induced oxidation (Figure S3), and the experimental assay yielded a total loss of 59.8 ± 14.4 μM NADH. Subtracting these values gives a final calculated concentration of 31.6 ± 8.2 μM loss of NADH (Figure 4B). A cYY calibration curve (Figure S2B) was then used to calculate the concentration of product yielded from the CYP121 reaction, which gave rise to 26.4 ± 1.9 μM (Figure 4D).

To confirm the presence of formaldehyde with a more specific assay, FDH was combined with NAD⁺ and added to the CYP121 reaction flow-through as described above. As seen in Figure 4C, the production of NADH (33.9 ± 3.2 μM) was observed when monitoring the absorbance at 339 nm when compared to the control reaction without formaldehyde.

Because of its ubiquitous presence, formaldehyde is notoriously difficult to analyze. Therefore, we employed a third method to quantifying formaldehyde formation.26 This confirmation method employed the known aldehyde and ketone coupling agent 2,4-dinitrophenylhydrazine (2,4-DNP).27 The formaldehyde-coupled product was observed as a new peak in HPLC (Figure S4), and a calibration curve was used to quantify the formaldehyde-coupled product (Figure S5), which yielded 35.7 ± 2.2 μM. Together, we conclude that an O-demethylation reaction instead of C−C bond coupling occurs when cYF-4-OMe is used in place of cYY as the substrate for CYP121.

Intermediate Crystal Structure Captured during in Crystallographic Reaction. To provide evidence that a hydroxylation takes place at the methyl group of the probe followed by an O-demethylation reaction, the cocrySTALLized CYP121-cYF-4-OMe crystals were soaked in a mother liquor at pH 7.4 with 20% glycerol. H₂O₂ was added, and crystals were soaked for 30 s to 10 min before being flash-cooled in liquid nitrogen. Among the X-ray diffraction datasets collected, a potential reaction intermediate was obtained in crystallo as described below, and it was not otherwise populated.

A putative intermediate was captured after soaking CYP121 cocrySTALLized with cYF-4-OMe with H₂O₂ for 2 min (Figure S) and refined to 1.81 Å resolution (Table 1). Initially, small
excess density was noticed, tailing off the methoxy group toward the heme that was not observed in the CYP121-cYF-4-OMe complex. Refinement with cYF-4-OMe did not present a full account for the unoccupied electron density connected to the methoxy group. Fitting the ligand lead to a 50:50 occupancy of cYF-4-OMe and cYF-4-OMeOH, which eliminated the excess density. The bond distances from the intermediate structure to Ser237, Arg386, and to the heme for both the probe and its intermediate structure are shown in Figure 6. The bond length from the iron ion to the cysteine-ligated residue changed from 2.4 Å in the cocrystallized structure to 2.6 Å in the proposed intermediate. Heme reflux was measured for both the cocrystallized complex and the putative intermediate as seen in Figure S6. The cocrystallized complex yielded angles of 163.2 and 178.5° compared to the oxidant soaking structure, which had angles of 163.0 and 177.4°. The excess density observed in the proposed intermediate structure coupled with the satisfying fitting of the electron density and the noticeable small changes in heme angles and distance to the cysteine-ligated residue are in accordance with a captured intermediate (Figure 6). It should be noted that this intermediate was not seen in the shorter or longer reaction times of the experiments.

**Determination of the C−O Bond Cleavage by 18O-Labeling Studies.** To further pinpoint the location of the C−O bond cleavage between an alkyl−O bond (as illustrated in Scheme 1B) or an aromatic carbon−O after the hydroxylation reaction, isotope labeling experiments were performed. 18O-labeled water and hydrogen peroxide were employed to identify whether cleavage occurs on the aryl side or the methyl side of the methoxy oxygen, respectively. Concentrated stock solutions of CYP121, peracetic acid, and Tris-HCl buffer (pH 7.4) were diluted using 18O-enriched water, which made up ≥85% of the final reaction solution. The product was collected and analyzed via high-resolution mass spectrometry (HRMS), which yielded an m/z of 327 with no 18O incorporation observed, indicating that the solvent was unlikely to participate in a nucleophilic attack on the aromatic ring after oxidation similar to what has been observed in the dehaloperoxidase mechanism.28 Next, 18O-labeled H218O2 was employed. A stock solution of H218O2 was made to 2 mM concentration and added to the reaction mixture in place of peracetic acid as previously described. Again, the product was collected and yielded the same m/z as has been observed (Figure S1B). This lack of 18O incorporation into the product indicates that after hydrogen atom abstraction, the ferryl-oxo intermediate does not insert itself directly into the aromatic ring but rather rebounds onto the methylene carbon, resulting in deformylation, consistent with the conclusion that C−O bond cleavage occurs on the methyl side of the methoxy oxygen. This is a
The evidence provided here supports a switch in CYP121 activity from C–C bond coupling of its native substrate to hydroxylation and then O-demethylation through oxygen atom insertion into cYF-4-OMe, resulting in a demethylation to release formaldehyde and cYY. By blocking a hydroxyl group of cYY in the probe, the peroxidase-like CYP121 exposes its raw activity for a P450 enzyme has only been previously observed in a few cases including P450 CYP1A2 and the newly described CYP255A (GcoA). The structural data obtained in this study show that the sole reason CYP121 performs the chemically easier hydrogen atom abstraction from the phenolic oxygen of cYY rather than electrophilic substitution of the C–H bond site next to the phenol is due to the substrate structure and positioning at the enzyme active site.

Because cYY is an observed product of the cYF-4-OMe reaction mediated by CYP121, the question of why mycocyclosin was not detected as a product naturally arises. During the peroxide shunt reaction with cYF-4-OMe, only a relatively small quantity of cYY would be formed, which is not kinetically competent to outcompete the large excess of cYF-4-OMe. The $K_D$ of cYF-4-OMe (12.9 ± 0.5 μM), compared to our reported $K_D$ of cYY (19.4 ± 0.6 μM), is in agreement with previously published data supports this conclusion. The continued exposure to peroxide, as previously shown, damages CYP121, hence reducing the amount of the catalyst and limits the amount of the cYY production in the peroxide shunt reaction. The results of this study provide the first experimental evidence for (1) the importance of the hydrogen atom on the hydroxyl group of cYY for mycocyclosin formation and (2) the rebound on the methyl group precedes deformylation. Thus, the mechanism of demethylation was investigated by product analysis and $^{18}$O isotope studies. A peroxidase-like mechanism would require a one- or two-electron oxidation of the 4-O-methyltyrosyl moiety by a high-valent iron species followed by the nuclophilic attack from solvent water or some iron-oxygen

**Figure 7.** pH dependence of the CYP121 reaction with cYY or cYF-4-OMe. Left, reaction of CYP121 and cYY using various buffers: 150 mM HEPES buffer (pH 7.4, black), 75 mM CHES buffer (pH 9.5, red), and 150 mM phosphate buffer (pH 10.4, blue). As pH increases, nearly full catalytic turnover is observed, forming the product mycocyclosin at 4.3 min. Right, the CYP121 reaction with cYF-4-OMe using the same set of the buffers.

### DISCUSSION

The observation of apparent demethylation of cYF-4-OMe by HPLC and HRMS could be rationalized by either a peroxidase-like reaction in which a tyrosyl cation radical is attacked by a solvent water molecule or a P450 reaction in which hydrogen atom abstraction followed by oxygen atom rebound on the methyl group precedes demethylation. Thus, the mechanism of demethylation was investigated by product analysis and $^{18}$O isotope studies. A peroxidase-like mechanism would require a one- or two-electron oxidation of the 4-O-methyltyrosyl moiety by a high-valent iron species followed by the nuclophilic attack from solvent water or some iron-oxygen...
species, resulting in release of methanol. However, when CYP121 reacted with cYF-4-OMe using either 18O-labeled water or peroxide, no isotope enrichment was observed in the product, cYY, eliminating a peroxidase-like mechanism in the CYP121 reaction with cYF-4-OMe. The P450 mechanism for demethylation, however, would not be expected to enrich 18O in the product, but rather, a compound I (Fe(IV) = O) species could initiate a radical reaction with the methyl group by abstraction of a hydrogen atom, forming compound II (Fe(IV)−OH) and leaving a radical on the methylene carbon. The −OH from compound II can then rebound to the methylene radical, forming the hydroxylated cYF-4-OMe intermediate, cYF-4-OMeOH, which can then undergo base-catalyzed C−O bond cleavage via deformylation. The observation of no isotope scrambling at the methoxy oxygen and quantitative formation of formaldehyde strongly supports the cytochrome P450 mechanism for O-demethylation of cYF-4-OMe.

From our proposed mechanism, the initial abstraction of a hydrogen atom from the methyl group by compound I and subsequent oxygen rebound ultimately, resulting in deformylation that indicates a hydroxylated probe, cYF-4-OMeOH, as an intermediate. Spontaneous deformylation has not been studied for O-methylphenols, although phenoxy 1-methanol has been used in synthetic chemistry; therefore, cYF-4-OMeOH may be stable enough to be captured, although the question arises of whether or not deformylation is enzymatic. Previously, O-dealkylations have been reported to occur from C−H bond hydroxylation of a methyl carbon, resulting in deformylation by other cytochrome P450 enzymes. Conversely, horseradish peroxidase utilizes an electron transfer mechanism, which causes loss of methanol instead, which is not observed for CYP121.

Further evidence for the oxygen-rebound mechanism of the compound II is found in the in crystallo reaction studies with H2O2. At 2 min of soaking of the CYP121-cYF-4-OMe complex crystals with H2O2, excess electron density is observed, which connects to the unassigned density on top of the Fe and the methyl carbon of cYF-4-OMe. Fitting the ligand density with cYF-4-OMeOH led to reasonable fitting of the extra density (Figure 5). In its native reaction, CYP121 is expected to deliver two oxidizing equivalents to cYY. However, steric hindrance of the L-shaped substrate-binding pocket precludes the C−C bond coupling of doubly oxidized cYY (i.e., cYY·cYY). The product mycocyclosin can only be formed during the exit of the postulated cYY·cYY intermediate. For this reaction, the product-bound structure has not become available either through cocrystallization, soaking, or in crystallo chemical reactions nor any catalytic intermediate after arrival of the oxidant to the ES complex. The cYF-4-OMeOH bound crystal structure of CYP121 represents the first intermediate of the enzyme. Since cYF-4-OMeOH is not anticipated to spontaneously decompose to cYY and formaldehyde under these conditions in the time scale comparable to the solution studies described above, the subsequent O-demethylation is expected

Scheme 2. Proposed Mechanistic Pathway of CYP121 Using cYF-4-OMe as an Alternate Substrate

“The O-demethylation of cYF-4-OMe is accomplished through a C−H bond activation and hydroxylation of the methyl group, followed by deformylation. The cocrystallized enzyme-probe complex and the enzyme-intermediate structures are marked with green and yellow carbons, respectively. The methoxy oxygen is colored blue and the methyl group is colored red to track the atoms throughout the mechanism.”
to be an enzymatic reaction mediated by CYP121 (Scheme 2) as described in CYP1A2 and CYP225A.

Due to the unusual, peroxidase-like reaction performed by CYP121 on cYY to form mycocyclosin as well as its ability to robustly perform peroxidase assays, the question arises as to what differentiates CYP121 from more typical cytochromes P450. One potential explanation is that the redox potential of the presumed compound I intermediate that initiates catalysis may be lower than normal so that only a phenolic hydrogen atom could be abstracted, preventing CYP121 from hydroxylating its substrate. This study unexpectedly gauged the atom could be abstracted, preventing CYP121 from hydroxylating its substrate. This study unexpectedly gauged the magnitude of the power of the enzyme-based oxidant. The observation of oxidative O-demethylation activity shows that the redox potential of the CYP121 compound I species suffices for abstraction of a hydrogen atom from carbon. Thus, the factors driving peroxidase-like activity in the native reaction must lie with the active site architecture or dynamics of the enzyme.

**CONCLUDING REMARKS**

As a potentially druggable enzyme that performs interesting chemistry, CYP121 is suitable for mechanistic investigation. In this study, a substrate analogue was synthesized to probe the reactivity of CYP121. Compared to the native substrate cYY, the asymmetric synthetic probe cYF-4-OMe has a very specific binding mode in CYP121. Unlike cYY, the presence of the methyl group causes a significant low- to high-spin shift upon binding, as observed by EPR, which is not observed for the native substrate. Such a shift is interpreted usually as a departure of the solvent-derived ligand to facilitate the oxidant binding to the iron ion. Various analytical and biochemical methods showed that CYP121 can perform O-demethylation via a more typical hydroxylation reaction in addition to its normal C–C bond-forming reaction. Mechanistic study confirmed that the demethylation takes place via alkyl hydroxylation of the methoxy group, followed by demethylation. The first intermediate of a P450-catalyzed O-demethylation was captured and characterized by X-ray crystallography, which is essential for understanding the O-demethylation mechanism mediated by P450 enzymes. This work highlights the importance of the hydroxyl hydrogen of cYY in the formation of mycocyclosin and establishes that CYP121 can mediate C–H bond activation typical of cytochromes P450 in addition to its native peroxidase-like reaction for C–C bond formation.

A common trait of heme enzymes is that they often form a high-valent iron-oxo intermediate, which acts as a powerful oxidant. While this species can mediate a remarkable array of oxidation reactions, it is generally true that a given enzyme promotes only a specific type of reaction. How the reaction type is determined after the formation of the key oxidant remains an open question, the answers to which bear on our fundamental understanding of enzyme catalysis as well as de novo enzyme design and protein engineering. The study described above indicates that the type of reaction catalyzed by CYP121 is not decided by the heme cofactor alone, and that the substrate plays a role in assisting in directing the oxidizing power to a specific type of chemical reaction. These findings provide insight into the mechanistic determinants of the enzyme for future drug development.

**MATERIALS AND METHODS**

**Chemicals.** N-Boc-O-methyl-L-tyrosine (98%) and L-tyrosine methyl ester hydrochloride (98%) were purchased from Alfa Aesar and used as received. All other chemicals were purchased from Sigma-Aldrich unless otherwise stated. A CombiFlash RF+ flash chromatography system was used for product purification.

**Synthesis of cYF-4-OMe.** This compound was synthesized as described with few modifications. N-Boc-O-methyl-L-tyrosine (443 mg, 1.5 mM) was dissolved in 7 mL of dimethylformamide in a 25 mL flask. The flask was charged with L-tyrosine methyl ester hydrochloride (348 mg, 1.5 mM), stirred until fully dissolved, and warmed to 60 °C. Disopropylethylamine (523 μL, 3.0 mM) and HATU (627 mg, 1.7 mM), purchased from Combi-Blocks, were added. The air in the flask was exchanged with argon and left to stir for 4 h. The reaction was quenched with ethyl acetate, washed with sodium bicarbonate and brine, and dried with sodium sulfate. The organic solvent was removed in vacuo, and the resulting cream-colored solid was purified with a flash column using a gradient of ethyl acetate in hexane from 5 to 85% over a period of 20 min. The product was collected in 5 mL test tubes and combined, and the solvent was removed in vacuo. The resulting white solid was then dissolved in formic acid (5 mL in vacuo. The resulting white solid was then dissolved in formic acid (5 mL mM M−1) and monitored via thin-layer chromatography until fully deprotected. The formic acid was removed in vacuo. The flask was charged with a mixture of sec-butanol and toluene (5:1) and heated to 105 °C for 2 h. The resulting crystallized white solid was filtered out and washed with ethyl acetate (85%, 434 mg, 1.3 mmol); [MH]+ m/z, 341.1339; 1H NMR (300 MHz, DMSO-d6): δ ppm 2.06–2.26 (m, 2H), 2.53–2.61 (m, 2H), 3.70 (s, 3H), 3.88 (br. s., 2H) 6.68 (m, J = 8.20 Hz, 2H), 6.85 (d, J = 8.20 Hz, 4H), 6.91–7.00 (m, 2H), 7.81 (d, J = 6.44 Hz, 2H), 9.21 (s, 1H).

**Enzyme Overexpression and Purification.** Expression and purification of CYP121 from Mtb has been described elsewhere. CYP121 was purified as described with minor modification. A starter culture was made using a 250 mL flask containing 150 mL of LB media culture mixed with kanamycin (100 μM). A starter culture Escherichia coli bearing the CYP121 expression plasmid was made from glycerol stock stored at −80 °C. An autoclaved flask with LB broth was inoculated and cultured for 24 h in an incubator at 220 rpm and 37 °C. The next day, the starter culture was used to inoculate 11 LB/kanamycin cultures (1 L), which were then grown at 220 rpm and 37 °C. The cultures were then pelleted by centrifugation (20,000 × g, 34,000 psi). The cell debris was then pelleted by centrifugation (2 × 34,000g for 40 min), and the lysate was loaded onto a Ni-NiNTA affinity column. Before loading, the column was prepared using 500 mM imidazole buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 5% glycerol) to remove nonspecifically bound protein. The column was re-equilibrated with buffer not containing imidazole, and the lysate was loaded onto the column (1.2
mL min⁻¹) and washed with buffer (1.5 mL min⁻¹). After the protein is fully loaded, 15 mM imidazole buffer was used to wash the column until a peak consisting of weakly bound protein is eluted. A gradient from 20 to 425 mM imidazole is used over 120 mL to elute CYP121. Five fractions were collected, the first two and last three pooled and concentrated separately using a 30 kDa membrane. The protein was then desalted using a Sephadex G-25 desalting column using 50 mM Tris-HCl (pH 7.4). The fractions containing protein were collected and concentrated, and protein and heme concentrations were determined using ε₂₅₀ = 26,500 M⁻¹·cm⁻¹ and ε₄₁₆ = 110,000 M⁻¹·cm⁻¹. The purified protein was concentrated to ∼700 μM, and heme occupancy averaged 55% [heme]/[protein] as determined by UV–vis. Purified protein was frozen in liquid nitrogen and stored at −80 °C until needed.

**Reaction Setup and HPLC Analysis.** Reactions and HPLC analysis were conducted at room temperature in 50 mM Tris-HCl (pH 7.4). CYP121-cYF-4-OMe was dissolved in DMSO to make a 27 mM stock solution. The stock of peracetic acid (2 mM) was made and titrated into the reaction mixture. CYP121 (50 or 500 μM) was incubated with cYF-4-OMe (600 μM) for 5 min before peracetic acid (300 μM) was titrated over a 15 min period to avoid rapid heme bleaching. Four controls were considered (i) CYP121 only, (ii) CYP121 and cYF-4-OMe, (iii) CYP121 and peracetic acid, and (iv) cYF-4-OMe and peracetic acid.

The enzyme was then filtered out using a 10 kDa concentrator (Millipore) at 15,000 × g over a period of 20 min. Fifty microliters of enzyme was injected onto an Inert Sustain C18 column (5 μM particle size, 4.6 × 100 mm, GL Sciences Inc.) and analyzed via a Thermo Scientific cUltimate-900 HPLC system. Measurements were made in a SHQE-W resonator at 6 K and 1.002 THz modulation amplitude. Data were collected at 10 K and 1.002 mW microwave power and 50 K and 0.200 mW microwave power.

**Secondary Product Identification.** Alcohol dehydrogenase from *Saccharomyces cerevisiae* (yeast) was purchased from Sigma-Aldrich as a lyophilized powder and used as received. ADH (~50 units) was mixed with 200 μM NADH and 400 μL of CYP121 reaction flow-through from the previous section. The absorbance was monitored at 339 nm using a UV–vis spectrophotometer (Agilent 8453) during the reaction.

Formaldehyde dehydrogenase from *Pseudomonas* was purchased from Sigma-Aldrich as a lyophilized powder and used as received. FDH (~0.5 units) was mixed with 200 μM NAD⁺ and CYP121 reaction flow-through, and the reaction was monitored as described above.

Chemical method of formaldehyde formation quantitation was also attempted. 2,4-Dinitrophenyhydrazine (2,4-DNP) was purchased from Sigma-Aldrich and prepared using previously published methods. The reaction of CYP121 with cYF-4-OMe was monitored as described above and heated to precipitate the enzyme, and then the solids were pelleted. The supernatant was collected, and 2,4-DNP was added to make up 1.5% of the mixture. The resulting mixture was then analyzed via HPLC, utilizing a gradient of 5–95% acetonitrile in H₂O with 0.1% formic acid.

**18O Incorporation Analyses.** 18O-water and 18O-hydrogen peroxide were purchased from Sigma-Aldrich. For 18O-H₂O₂ experiments, the reaction setup is as described above. H₂₁₈O₂ (300 μM) was titrated into a solution of CYP121 (50 μM) and cYF-4-OMe (700 μM) in 50 mM Tris-HCl (pH 7.4) over a period of 15 min. The enzyme was then removed via a 10 kDa concentrator (Millipore). Analytes were separated on HPLC, and the product was collected and analyzed via HRMS.

For the 18O water experiments, a 1 M buffer solution (50 mM Tris-HCl, pH 7.4) and stock solution of peracetic acid were made by diluting with isotopically labeled water where the 18O water did not make up less than 85% of the total solution. From this, the reaction was conducted and analyzes as previously described.

**pH Dependence Study.** The following buffers were used for pH-dependent experiments: 150 mM HEPES buffer (pH 7.4), 75 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES, pH 9.5), and 150 mM phosphate buffer (pH 10.4).

**Crystallization, in Crystallo Chemical Reaction, X-Ray Data Collection, and Data Analyses.** Crystals of the CYP121-cYF-4-OMe complex were obtained using the hanging drop method. The N-terminal His₆-tagged enzyme was cleaved using a Thrombin CleanCleave Kit (Sigma-Aldrich) prior to crystallization. The enzyme buffer consisted of 50 mM Tris-HCl (pH 7.4). Crystallization conditions consisted of 100 mM MES (pH 5.0−5.7) and 1.85−2.05 M ammonium sulfate with 600 μM CYY or 400 μM cYF-4-OMe at 6 °C in a vibration-free crystallization refrigerator. The protein was mixed in a 1:1 ratio with the crystallization solution for a total volume of 2 μL, and the crystals began appearing after 1.5 weeks.

To increase the pH of the crystals to 7.4, four incremental solutions of 100 mM MES (pH 5.9−7.4) were made with 2.0 M ammonium sulfate. Crystals were soaked in increasing pH crystallization solutions for 20 min until pH 7.4. To obtain an intermediate complex, crystals were picked and placed into 25 μL of cryoprotectant containing 100 mM MES (pH 7.4), 2.0 M ammonium sulfate, and 20% glycerol. H₂O₂ was added to the cryoprotectant at a final concentration of 5 mM and gently mixed. Crystals were mounted onto loops and stored in liquid nitrogen at various times ranging from 30 s to 10 min.

X-ray diffraction data were collected at the beamline 9-2 of the Stanford Synchrotron Radiation Lightsource (SSRL), National Accelerator Laboratory (SLAC) for the cocrystallization complex and at Argonne National Laboratory Advanced Photon Source (APS) beamline 19-BM. Diffraction data were collected at a 1.0 Å wavelength with a Dectris Pilatus 6M detector (SSRL) or ADSC Quantum 315r detector (APS), and the crystal was kept under a nitrogen stream at 100 K. The data were processed with HKL3000. The structure was solved using Phaser-MR as the search template and refined in the Phenix program package. Ligands including heme and cYF-4-OMe for...
the cocrystallization structure were observed at 100% occupancy within the active site after data collection, molecular replacement, and refinement. H2O2 soaking experiments were observed at 98% occupancy after refinement. Water molecules were added during the final refinement, and WinCoot39 and PyMOL39 were used for structure modeling.

## Associated Content

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.9b04596.

Synthetic scheme for cYF-4-OMe (Scheme S1), 3H NMR and high-resolution mass spectrometry (Figure S1) of the synthetic probe, calibration curves of cYF and NADH (Figure S2), alcohol dehydrogenase control with NADH (Figure S3), HPLC chromatogram of the quantitation of formaldehyde as a secondary product in the reaction of CYP121 with cYF-4-OMe (Figure S4), and calibration curve for the coupling reaction of 2,4-DNP (Figure S5), measured heme ruffle of CYP121 cocrystallized with cYF-4-OMe compared to the captured intermediate cYF-4-OMeOH (Figure S6), and analysis of the binding affinity of CYP121 with cYF-4-OMe (Figure S7) (PDF)

### Author Information

**Corresponding Author**

Aimin Liu — University of Texas, San Antonio, Texas; orcid.org/0000-0002-4182-8176; Phone: +1-210-458-7062; Email: Feradical@utsa.edu; Fax: 210-458-7428

**Other Authors**

Romie C. Nguyen — University of Texas, San Antonio, Texas; Yu Yang — University of Texas, San Antonio, Texas; Yifan Wang — University of Texas, San Antonio, Texas; orcid.org/0000-0002-1566-4972

Complete contact information is available at: https://pubs.acs.org/10.1021/acscatal.9b04596

### Notes

The authors declare no competing financial interest.

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### References


Supporting Information

Substrate-Assisted Hydroxylation and O-Demethylation in the Peroxidase-like Cytochrome P450 Enzyme CYP121

Romie C. Nguyen, Yu Yang, Yifan Wang, Ian Davis, and Aimin Liu*

Department of Chemistry, University of Texas, San Antonio, Texas 78249, United States

* Feradical@utsa.edu

Scheme S1. The synthetic scheme for cYF-4-OMe
Figure S1A. $^1$H-NMR spectrum of cYF-4-OMe in DMSO-d$_6$.

Figure S1B. HRMS analysis of cYF-4-OMe (left) and the collected reaction mixture product (right) which eluted at ~6.3 mins on HPLC. 50 mM Tris-HCl pH 7.4 buffer was used in the reaction mix.
Figure S2A. Calibration curve of NADH

Figure S2B. Calibration curve of cYY.
**Figure S3.** The ADH control experiment with NADH present. The loss of NADH was monitored over the course of 33 minutes. Black trace represents the initial measurement and red trace is the final measurement.
Figure S4. HPLC chromatogram of the quantitation of formaldehyde as a secondary product in the reaction of CYP121 with cYF-4-OMe by using 2, 4-dinitrophenylhydrazine. The CYP121 reaction with cYF-4-OMe (navy blue) shows cYY elution occurring at 4 min and cYF-4-OMe elution at 5.4 min. The control for 2, 4-DNP in buffer (black) indicates that the reagent elutes at 7.3 min with a small amount of coupled product eluting at 8.8 min. PAA and 2, 4-DNP (magenta) and cYF-4-OMe and 2, 4-DNP (green) controls are also shown. To quantify formaldehyde, 2, 4-DNP was added to the CYP121 and cYF-4-OMe reaction mixture (red) and then separated, showing the formaldehyde-coupled product eluting at 8.8 min.
Figure S5. Calibration curve for the coupling reaction of 2,4-DNP with varying concentrations of formaldehyde using HPLC.
Figure S6. Measured heme ruffle of CYP121 co-crystallized with cYF-4-OMe (left) compared to the captured intermediate cYF-4-OMeOH (right).
Figure S7. Analysis of the binding affinity of CYP121 with cYF-4-OMe.