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A Single-Site Mutation at Ser146 Expands the Reactivity of the Oxygenase Component of *p*-Hydroxyphenylacetate 3-Hydroxylase

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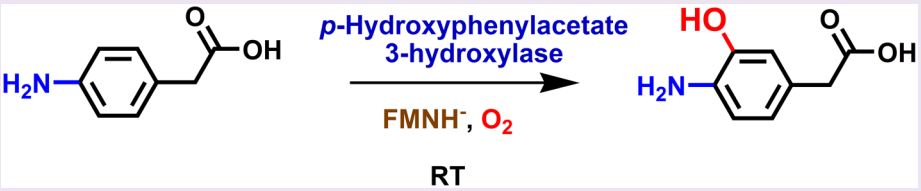
Supporting Information



ABSTRACT: The oxygenase component (C_2) of *p*-hydroxyphenylacetate (4-HPA) 3-hydroxylase (HPAH) from *Acinetobacter baumannii* catalyzes the hydroxylation of various phenolic acids. In this report, we found that substitution of a residue close to the phenolic group binding site to yield the S146A variant resulted in an enzyme that is more effective than the wild-type in catalyzing the hydroxylation of 4-aminophenylacetate (4-APA). Product yields for both wild-type and S146A enzymes are better at lower pH values. Multiple turnover reactions of the wild-type and S146A enzymes indicate that both enzymes first hydroxylate 3-APA to give 3-hydroxy-4-aminophenylacetate (3-OH-4-APA), which is further hydroxylated to give 3,5-dihydroxy-4-aminophenylacetate, similar to the reaction of C_2 with 4-HPA. Stopped-flow experiments showed that 4-APA can only bind to the wild-type enzyme at pH 6.0 and not at pH 9.0, while it can bind to S146A under both pH conditions. Rapid-quench flow results indicate that the wild-type enzyme has low reactivity toward 4-APA hydroxylation, with a hydroxylation rate constant (k_{OH}) for 4-APA of 0.028 s^{-1} compared to 17 s^{-1} for 4-HPA, the native substrate. In contrast, for S146A, the hydroxylation rate constants for both substrates are very similar (2.6 s^{-1} for 4-HPA versus 2.5 s^{-1} for 4-APA). These data indicate that Ser146 is a key catalytic residue involved in optimizing C_2 reactivity toward a phenolic compound. Removing this hydroxyl group expands C_2 activity toward a non-natural aniline substrate. This understanding should be helpful for future rational engineering of other two-component flavin-dependent monooxygenases that have this conserved Ser residue.

Flavin-monooxygenases are important biocatalysts.[1,2] These enzymes can catalyze the regio-specific incorporation of a single oxygen atom in the form of a hydroxyl or epoxide group into a wide range of substrates. Examples of enzymes in this class that are useful in biocatalysis include Baeyer−Villiger monooxygenases, styrene monooxygenase, and various aromatic hydroxylases.[3−5] As biocatalysts are instrumental for the development of green and sustainable manufacturing processes, the engineering of biocatalysts to broaden their repertoire of natural enzymatic activities has the potential to provide a comprehensive enzymatic toolbox to fit a variety of applications.[6] The expansion of enzymatic specificity to encompass reactivity toward non-native substrates is generally accomplished by engineering enzymes through a random, semirandom, or rational approach.[6−8] The rational engineering approach, which is the most cost-effective method, requires an understanding of the catalytic reaction mechanism and of how the reaction reactivity and specificity is controlled by active site residues.

Most of the flavin-dependent monooxygenases that can hydroxylate aromatic compounds require the presence of a phenolic group on the substrate to facilitate the electrophilic aromatic substitution reaction.[5,9,10] In addition to phenolic compounds, a few enzymes can hydroxylate aniline compounds such as the $FADH_2$-utilizing monooxygenase (GTNG_3160) encoded in the anthranilate degradation gene cluster in *Geobacillus thermodenitrificans* NG80-2 which catalyzes the conversion of anthranilate (or 2-aminobenzoate) into 3-hydroxyanthranilate.[11] Some hydroxylases that can use aniline derivatives such as 4-aminophenylalkanoic acid or 4-aminobenzoic acid as substrates have combined activities of hydroxylation with either deamination or decarboxylation.[12−14] *p*-Hydroxybenzoate hydroxylase from *P. fluorescens*[15] and *P. aeruginosa*[16] can catalyze *ortho*-hydroxylation of aniline

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derivatives (*p*-aminobenzoate) but with much smaller hydroxylation rate constants (more than 10-fold) compared to those for the native phenolic substrates. The hydroxylation of 4-aminophenylalkanoic acid to produce the corresponding 3-hydroxy-4-aminophenylalkanoic acid is potentially useful in biocatalysis because many of these compounds, such as 3-hydroxy-4-aminophenylacetic acid (3-OH-4-APA), are precursors for the synthesis of pharmacologically active compounds.[17−22]

p-Hydroxyphenylacetate 3-hydroxylase (HPAH) from *Acinetobactor baumannii* makes a good candidate starting enzyme for exploratory engineering to produce an enzyme that can prepare 3-hydroxy-4-aminophenylacetate (3-OH-4-APA) from 4-aminophenylacetate (4-APA). Native HPAH catalyzes *ortho*-hydroxylation of *p*-hydroxyphenylacetate (4-HPA) to form 3,4-dihydroxyphenylacetate (3,4-DHPA). The enzyme is a two-component flavin-dependent monooxygenase that consists of reductase (C_1) and oxygenase (C_2) components.[23,24] The reaction mechanism of C_2 is one of the best understood among the two-component flavin-dependent monooxygenases,[25−32] and X-ray crystal structures are available.[33] In addition to 4-HPA, C_2 can also catalyze the *ortho*-hydroxylation of other phenolic acids with R groups such as −COOH, $-CH_2COOH$, $-CH_2CH_2COOH$, and *trans*-CH═CHCOOH.[34] C_2 has many properties that make it suitable for biocatalytic applications including having a high yield of overexpression and exhibiting good stability over a wide-range of pH conditions (pH 6−10)[28] and temperatures (25−40 °C for more than 24 h).[34] Therefore, expansion of the activity of C_2 to go beyond hydroxylation of phenolic acids will make this flavin-monooxygenase a versatile enzyme useful for inclusion in a biocatalytic toolbox for multiple applications.

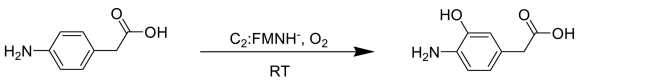
In this report, we found that engineering of C_2 at Ser146 yielded the S146A variant that is much more effective than the wild-type enzyme in catalyzing *ortho*-hydroxylation of 4-aminophenylacetic acid (4-APA) to generate 3-hydroxy-4-aminophenylacetic acid (3-OH-4-APA). The kinetics of ligand binding to wild-type and S146A toward 4-APA and 4-HPA were shown to be different. Measurement of the hydroxylation rate constants of the wild-type and S146A enzymes revealed that, while the wild-type enzyme has a much greater reactivity toward 4-HPA, the reactivity of S146A toward 4-APA and 4-HPA are not different. Overall, we have shown that a single-site mutation at S146 can expand the activity of C_2 to go beyond the natural phenolic acid substrate to include an aniline compound.

RESULTS AND DISCUSSION

Rational engineering of C_2 was performed in order to obtain a variant that is superior to the wild-type enzyme in catalyzing hydroxylation of 4-APA. Transient kinetics (stopped-flow and rapid-quench flow techniques) indicated that the hydroxyl group of Ser146 is key to specifying C_2 reactivity toward a phenolic compound. Hydroxylation of 4-APA by wild-type and S146A is more efficient at lower pH. Density functional theory calculations of the binding energy indicate that ligand binding is more favorable when 4-APA or His120 is protonated.

Single Turnover Reactions of Wild-type Enzyme with 4-APA at pH 7.0. Single turnover reactions of wild-type C_2 with 4-APA and other aromatic compounds with different types of substituents were carried out at pH 7.0 (see Methods). We chose to use single turnover reactions to identify the hydroxylation product in order to avoid complicating factors from the coupled enzymes (if multiple turnover reactions were employed). Under these conditions, the enzyme only catalyzed one catalytic cycle. Because the complex of C_2 and reduced FMN (C_2:$FMNH^-$) was the limiting reagent, it was oxidized in the excess of oxygen and could not be regenerated in the absence of reducing agent. The results indicated that 4-mercaptophenylacetate, 4-methoxyphenylacetate, 4-nitrophenylacetate, 4-fluorophenylacetate, and 4-chlorophenylacetate could not be used as substrates (data not shown) and only 4-APA could be used (Table 1). HPLC/DAD/MSD chromatograms of the wild-type C_2 reaction with 4-APA are shown in Supporting Information Figures S2−S4. The peak for the product as detected by HPLC/MSD-positive mode at 12.88 min showed an m/z of 168 (Supporting Information Figure S2d), and the peak for the substrate at 15.12 min showed an m/z of 152 (Supporting Information Figure S2c). The data indicate that the product has an m/z value of 16 AUs greater than the substrate (m/z 152), suggesting that the product is the hydroxylated 4-APA. This product has the same HPLC and mass spec profiles as the standard 3-hydroxy-4-aminophenylacetic acid (3-OH-4-APA; Supporting Information Figure S5), indicating that the wild-type C_2 catalyzes *ortho*-hydroxylation of

Table 1. Product Formation from Single Turnover Reactions[a] of Wild-Type C_2, Ser146, and His120 Variants with 4-APA (0.2 mM) at pH 6.0−9.0



4-Aminophenylacetic acid (4-APA) — 3-hydroxy-4-aminophenylacetic acid (3-OH-4-APA)

C_2 enzyme	pH	% yield of 3-OH-4-APA[b]
wild-type	6	40 ± 1
	7	31 ± 5
	8, 9	
S146A	6	100.0 ± 0.1
	7	95 ± 3
	8	77 ± 2
	9	66 ± 5
S146C	6	28.7 ± 0.8
	7	9 ± 3
	9	
H120N	6	17.1 ± 0.1
	7, 9	
H120D	6, 7, 9	
H120E	6, 7, 9	
H120K	6, 7, 9	
control (no enzyme)	6, 9	

[a]Under this experimental condition, the enzyme could only perform one catalytic cycle because C_2:$FMNH^-$ (limiting reagent) was all oxidized in the excess of oxygen. With no excess reducing agent, $FMNH^-$ could not be regenerated. The reactions were performed by mixing a 250 μL solution of C_2 (200 μM):$FMNH^-$ (100 μM) in 10 mM NaH_2PO_4 at pH 7.0 with a 250 μL solution of 400 μM substrate in various aerobic buffers including 100 mM NaH_2PO_4 at pH 6.0 and pH 7.0, 100 mM Tris-HCl at pH 8.0, and 100 mM Gly-NaOH at pH 9.0. The reactions were left at RT for 15 min and then quenched with 500 μL of 0.15 M HCl. The quenched solutions were filtered through Microcon centrifugal units (10 kDa cutoff), and the filtrates obtained were analyzed using HPLC/DAD/MSD. [b]The % yield of product was calculated from the amount of substrate consumed compared to the limiting agent (reduced flavin) in the reaction.

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ACS Chem. Biol. XXXX, XXX, XXX−XXX

4-APA to form 3-OH-4-APA (product identity was also confirmed by NMR, discussed below). Table 1 indicates that only a 31 ± 5% yield of 3-OH-4-APA was generated from the single turnover reaction at pH 7.0. The control reaction in the absence of C_2 did not show a peak at 12.88 min (Supporting Information Figure S3a and d).

Reactions of His120 and Ser146 Variants with 4-APA. The results of the single turnover reaction above indicate that wild-type C_2 is not an efficient biocatalyst for the hydroxylation of 4-APA. To improve the ability of C_2 to hydroxylate 4-APA, the residues located close to the binding site of the phenolic moiety were subjected to enzyme engineering. On the basis of the X-ray structure of the C_2:FMNH$^-$:4-HPA complex (PDB code 2JBT),[33] the hydroxyl group of 4-HPA is located close to His120 and Ser146—2.8 Å (Figure 1a). A previous report[30]

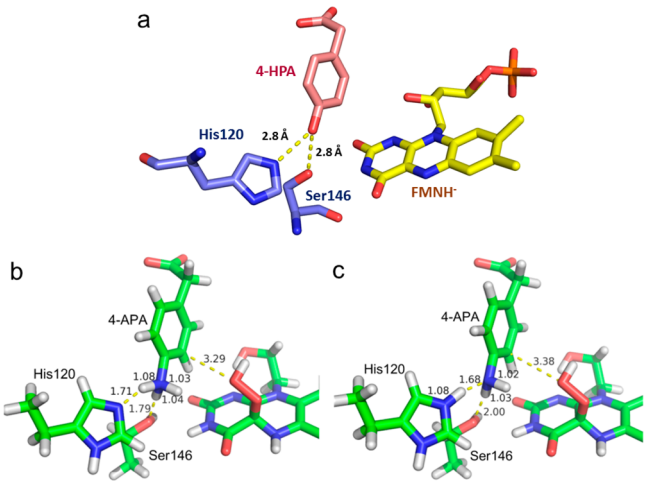

Figure 1. (a) Active site structure of the C_2:FMNH$^-$:4-HPA complex (PDB code 2JBT).[33] The optimized geometries of N-protonated 4-APA and neutral His120 (b) and neutral 4-APA and protonated His120 (c) in the active site model in the form of C_{4a}-hydroperoxyflavin. Bond distances are shown in Ångströms.

demonstrated that His120 and Ser146 are important for the hydroxylation of 4-HPA. His120 in particular may help in stabilizing the negative charge of the phenolate anion during the electrophilic aromatic substitution reaction. Mutation of Ser146 to Ala or Cys decreased the hydroxylation yield of 4-HPA to ~60−70% compared to the ~100% yield of the wild-type enzyme. As 4-APA has an amino group in place of the hydroxyl group of 4-HPA, we thought that production of variants with substitutions at His120 or Ser146, which are residues close to the phenolic binding site, might provide enzymes that are superior to the wild-type enzyme in the hydroxylation of 4-APA.

Several His120 and Ser146 variants with substitutions with negatively charged (H120D and H120E), positively charged (H120K), and neutral (H120N, S146C, S146A) residues were explored for their ability to perform 4-APA hydroxylation. Single turnover reactions of these His120 and Ser146 variants with 4-APA at pH 7.0 were carried out, and the results are shown in Table 1. The H120N, H120D, H120E, and H120K variants could not hydroxylate 4-APA, while S146C was able to hydroxylate 4-APA with a low product yield of 9 ± 3% hydroxylation. Among all of the variants explored, S146A was the most effective enzyme in catalyzing hydroxylation, resulting in a yield (~95 ± 3% yield) greater than that of the wild-type enzyme (~31 ± 5%). The chromatographic and MSD characteristics of the product from the S146A reaction with 4-APA are the same as those of the product from the wild-type enzyme (Supporting Information Figure S4a−d).

Reactions of Wild-type C_2 and His120 and Ser146 Variants with 4-APA at Various pH's. Single turnover reactions of wild-type C_2, S146A, S146C, H120E, H120K, and H120N with 4-APA (0.2 mM) were carried out over a pH range of 6.0 to 9.0 (see Methods), and the amount of product formed by each variant is shown in Table 1. H120D, H120E, and H120K were not able to catalyze the hydroxylation of 4-APA at any of these pH values, while the others were more effective at the lower pH values. For most enzymes, the hydroxylation yield of 4-APA is greater at lower pH's, and they cannot catalyze hydroxylation of 4-APA above pH 7.0. H120N hydroxylates 4-APA only at pH 6.0. Wild-type C_2 and S146C hydroxylates 4-APA only at pH 6.0 and 7.0. S146A, the enzyme with the highest efficiency of 4-APA hydroxylation, was able to catalyze the hydroxylation of 4-APA over a wide pH range (6.0−9.0). Based on these results, S146A was chosen for further investigations by transient kinetics to compare with the wild-type C_2.

Multiple Turnover Reactions of Wild-Type C_2 and S146A with 4-APA at pH 6.0. Multiple turnover reactions of wild-type C_2 and S146A with 4-APA at pH 6.0 (Supporting Information Scheme S1a) were carried out to investigate the bioconversion of 4-APA by these enzymes on a larger scale. The results show that both enzymes first catalyze hydroxylation of 4-APA to form 3-OH-4-APA. The 3-OH-4-APA was then hydroxylated to form 3,5-dihydroxy-4-aminophenylacetic acid (3,5-diOH-4-APA; Supporting Information Figure S6, Tables S1 and S2, and Scheme S1b). Structures of the single and double hydroxylated products were identified using ^{1}H and ^{13}C NMR spectroscopy (Supporting Information Figures S7−S10). Results of the multiple turnover reactions also indicate that the reaction of S146A with 4-APA or 3-OH-4-APA proceeds much faster than the reaction of the wild-type enzyme. The abilities of wild type and S146A to catalyze double hydroxylation of 4-APA is similar to the reactions of wild-type C_2 and Y398S with 4-HPA and *p*-coumaric acid,[34] in which the double hydroxylated product is the final product.

Transient Kinetic Analysis of Wild-Type C_2 and S146A Reactions to Investigate the Determining Factors that Make S146A More Efficient than the Wild-Type Enzyme in 4-APA Hydroxylation. The results in the sections above indicate that S146A is more efficient than the wild-type enzyme in the hydroxylation of 4-APA, especially at higher pH's (Table 1). We carried out transient kinetic experiments to test what catalytic steps in the reaction were affected by the substitution.

A. Reaction of Wild-Type C_2 and S146A:FMNH$^-$ with Oxygen in the Absence of Substrate at pH 6.0 and pH 9.0 Observed by Stopped-Flow Spectrophotometry. In the absence of substrate, the reactions of C_2:FMNH$^-$ or S146A:FMNH$^-$ with oxygen can only result in the formation of C_{4a}-hydroperoxyFMN and the subsequent elimination of H_2O_2 to give oxidized FMN (Scheme 1a).[28,30] The rate constants of C_{4a}-hydroperoxy-FMN formation were calculated from the first phase of the absorbance increase at 390 nm and at 446 nm, which indicate the formation of oxidized flavin (blue kinetic traces in Figures 2a,b and 3a,b, Supporting Figures S11a,b and S12a,b; Table 2). Overall, the rate constants associated with the two steps in both enzymes (Scheme 1a and

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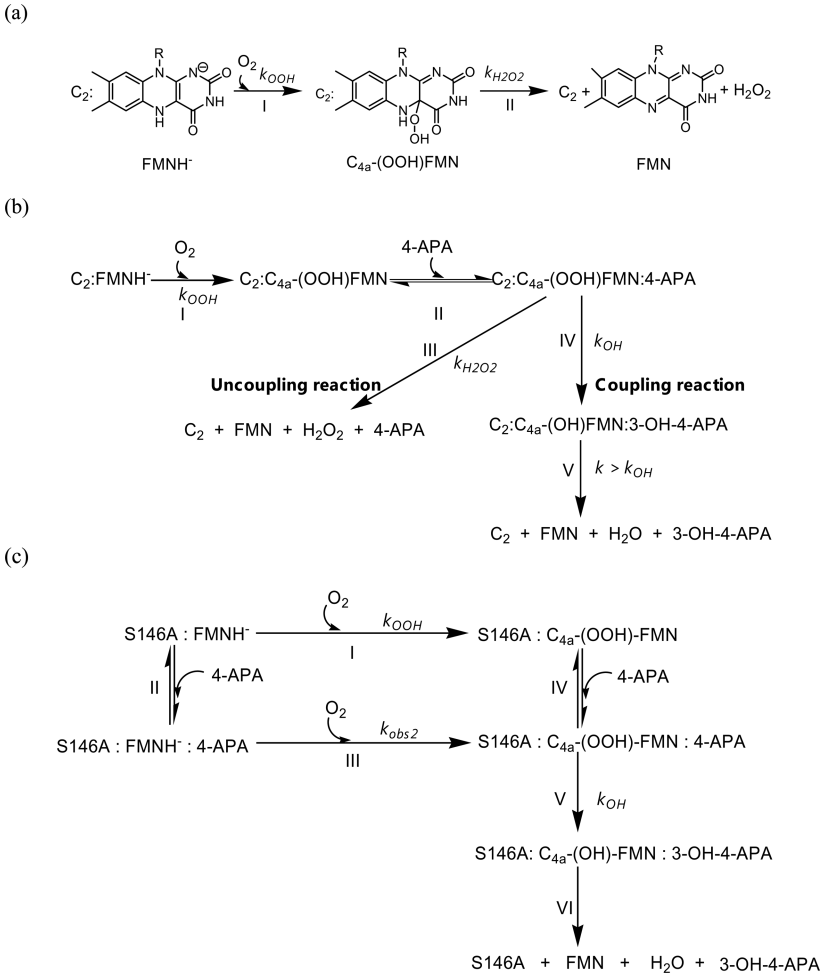
ACS Chem. Biol. XXXX, XXX, XXX−XXX

Scheme 1. (a) Reaction of Wild-Type C_2 or S146A with Oxygen in the Absence of 4-APA at pH 6.0 or 9.0, (b) Reaction of Wild-Type C_2 with Oxygen in the Presence of 4-APA at pH 6.0 or the Reaction of S146A with Oxygen in the Presence of 4-APA at pH 9.0,[a] (c) Reaction of S146A with Oxygen in the Presence of 4-APA at pH 6.0[b]



[a]The rate constant of step V is thought to be faster than the hydroxylation rate (k_{OH}) because the last phase of the stopped-flow results (flavin oxidation) occurred concomitantly with product formation (hydroxylation, rapid-quench flow results; Figure 2a and Supporting Figure S12a). [b]Rate constants are described as k_{OOH} = oxygen reaction, $k_{H_2O_2}$ = H_2O_2 elimination, and k_{OH} = hydroxylation.

Table 2) agree well with the results reported in previous studies.[28,30]

B. Reaction of Wild-Type C_2:FMNH⁻ with Oxygen in the Presence of 4-APA at pH 9.0 Observed by Stopped-Flow Spectrophotometry. As the reaction of the wild-type C_2 with 4-APA at pH 9.0 did not give any hydroxylation product (Table 1), we performed stopped-flow experiments similar to the experiments in section A, but in the presence of 4-APA (2−10 mM) to investigate whether 4-APA can bind to the wild-type enzyme at this pH. Kinetic traces of the reaction monitored at 390 and 446 nm shown in Supporting Figure S11a (black traces) indicate that the reactions of wild-type enzymes in the presence of 4-APA even at 10 mM are very similar to the reaction in the absence of 4-APA (blue traces). These data suggest that at pH 9.0, 4-APA has essentially no effect on the reaction of the reduced wild-type enzyme with oxygen, implying that it cannot bind to the wild-type enzyme. Therefore, the lack of 4-APA hydroxylation at this pH is likely due to the lack of 4-APA binding or binding of 4-APA in an unproductive manner, which does not disrupt the kinetics of C_{4a}-hydroperoxyflavin formation and decay.

C. Reaction of Wild-Type C_2:FMNH⁻ with Oxygen in the Presence of 4-APA at pH 6.0 Observed by Stopped-Flow Spectrophotometry. In contrast to the reaction of the wild-type C_2 and 4-APA at pH 9.0, the presence of 4-APA at pH 6.0 increases the observed rate constant of the second phase of the reaction to be much faster than the reaction performed in the absence of 4-APA (k_{reox} of 0.039 ± 0.001 s^{-1} versus ∼0.0018 s^{-1}, Figure 2a, black and blue kinetic traces). To address whether 4-APA can bind to a binary complex of C_2:FMNH⁻ before the enzyme reacts with oxygen, kinetic traces of the reaction of C_2:FMNH⁻ (syringe A) mixed with an oxygenated solution of 4-APA (syringe B) in the stopped-flow instrument were compared to the reaction of premixed C_2:FMNH⁻ and 4-APA (syringe A) with oxygenated buffer (syringe B). The results show that 4-APA does not affect formation of the C_{4a}-hydroperoxy-FMN even at 10 mM 4-APA (data not shown), implying that the ligand binds to the wild-type enzyme only after the intermediate is formed (Scheme 1b, step II). As the wild-type C_2 can hydroxylate 4-APA (2 mM) at pH 6.0 with ∼83 ± 0% yield (Table 2), the data indicate that the second phase observed in Figure 2a is comprised of multiple steps including binding of 4-APA to C_{4a}-hydroperoxyflavin, the

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ACS Chem. Biol. XXXX, XXX, XXX−XXX

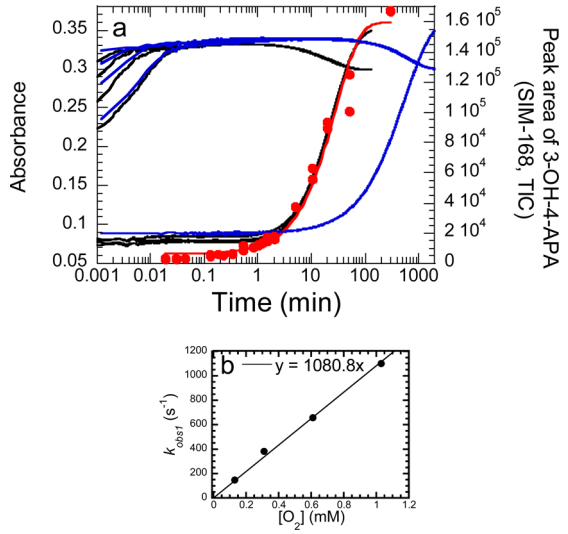

Figure 2. (a) Transient kinetics of the reaction of wild-type C_2 with oxygen in the presence of 4-APA at pH 6.0. A solution of wild-type C_2 (110 μM):$FMNH^-$ (50 μM) premixed with 4-APA (2 mM) was mixed with solutions of 0.26, 0.62, 1.22, and 2.06 mM oxygen and 4-APA 2 mM (before-mixing concentrations) in 100 mM NaH_2PO_4 at pH 6.0 at 4 °C (black kinetic traces). Reactions were monitored at 390 and 446 nm using a stopped-flow spectrophotometer. A plot of peak areas (SIM-168, TIC) of the hydroxylated product 3-OH-4-APA obtained from the rapid-quench flow experiments under the same conditions is shown in red filled circles. A single exponential fit of the data is shown by the red solid line, consistent with an observed hydroxylation rate constant (k_{OH}) of 0.034 s^{-1}. For reference, kinetics of the same reaction without 4-APA at pH 6.0 are shown by the blue traces. (b) A plot of the observed rate constants of the first phase (k_{obs1}) versus oxygen concentration. A bimolecular constant (k_{OOH}) from this plot was determined as 1.1 × 10^6 M^{-1} s^{-1}.

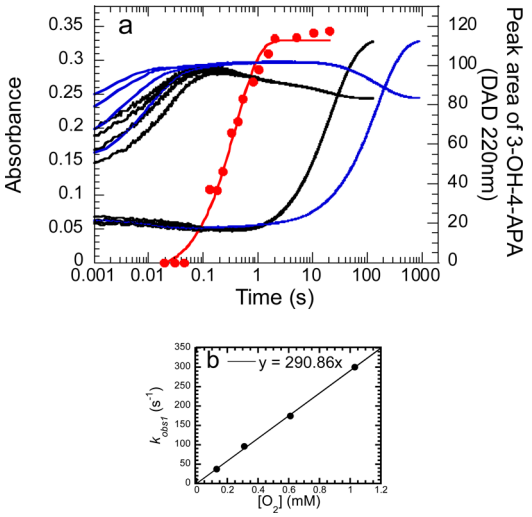

Figure 3. (a) Transient kinetics of the reaction of S146A with oxygen in the presence of 4-APA at pH 6.0 (black kinetic traces). A solution of S146A (110 μM):$FMNH^-$ (50 μM) premixed with 4-APA (2 mM) was mixed with solutions of 0.26, 0.62, 1.22, and 2.06 mM oxygen and 4-APA 2 mM in 100 mM NaH_2PO_4 at pH 6.0. Concentrations are as before mixing. Reactions were monitored at 390 and 446 nm using the stopped-flow spectrophotometer. A plot of peak areas (DAD 220 nm) of the hydroxylated product 3-OH-4-APA obtained from the rapid-quench flow experiments under the same conditions is shown by the red filled circles. A single exponential fit of the data is shown by the red line, consistent with an observed hydroxylation rate constant (k_{OH}) of 2.5 ± 0.2 s^{-1}. For reference, kinetics of the same reaction without 4-APA at pH 6.0 are shown in blue. (b) A plot of the observed rate constants of the first phase (k_{obs1}) versus oxygen concentrations. A bimolecular constant (k_{OOH}) from this plot was determined as 2.9 × 10^5 M^{-1} s^{-1}.

uncoupling reaction to eliminate H_2O_2, the coupling reaction to form the hydroxylated product, and dehydration to form the oxidized enzyme (Scheme 1b, steps II, III, IV, and V).

D. Measurement of the Hydroxylation Rate Constant of the Reaction of Wild-Type C_2 and 4-APA at pH 6.0 Using Rapid-Quench Flow Techniques. In order to directly measure the rate constant associated with the hydroxylation of 4-APA, rapid-quench experiments were carried out to measure the amount of 3-OH-4-APA product formed over time. The observed rate constant of hydroxylation (k_{OH}) was determined as ~0.034 ± 0.004 s^{-1} (Figure 2a, red filled circles and red line). Because the reaction undergoes bifurcation pathways of coupling and uncoupling reactions (Scheme 1b), the observed hydroxylation rate constant is the summation of the rate constants from both pathways as represented by eq 1. Based on a product ratio of 83% (Table 2), the actual rate constant of hydroxylation (k_{OH}) and H_2O_2 elimination ($k_{H_2O_2}$) under these conditions can be calculated as 0.028 s^{-1} and 0.006 s^{-1}, respectively (Table 2).

$$\text{ratio of product} = \frac{k_{OH}}{k_{obs}} = \frac{k_{OH}}{k_{OH} + k_{H_2O_2}} \quad (1)$$

E. Reaction of S146A:$FMNH^-$ with O_2 in the Presence of 4-APA at pH 9.0 Observed by Stopped-Flow Spectrophotometry. Unlike wild-type C_2 at pH 9.0 (section B), S146A can bind 4-APA at this pH. The presence of 4-APA increased k_{reox} of the second phase of the S146A reaction with oxygen from 0.028 ± 0.001 s^{-1} to ~1.64 ± 0.01 s^{-1} but did not affect the first kinetic phase (black versus blue traces, Supporting Figure S12a, Table 2). These results indicate that 4-APA can bind to S146A only after C_{4a}-hydroperoxyflavin formation (Scheme 1b, step II). The second observed phase was comprised of multiple steps, including the binding of 4-APA to C_{4a}-hydroperoxyflavin, the uncoupling reaction to eliminate H_2O_2 with $k_{H_2O_2}$ = 0.24 s^{-1} (Table 2), the coupling reaction to form the hydroxylated product with k_{OH} = 1.07 s^{-1} (Table 2), and the dehydration reaction to yield the oxidized enzyme (Scheme 1b, steps II, III, IV, and V).

F. Reaction of S146A:$FMNH^-$ with O_2 in the Presence of 4-APA at pH 6.0 Monitored by Stopped-Flow Spectrophotometry. The kinetics of the reaction of S146A:$FMNH^-$ with O_2 in the presence of 4-APA at pH 6.0 resulted in four phases (Figure 3a, black traces). Unlike the reaction of S146A at pH 9.0, the first and second phases are dependent on oxygen, implying that these two phases are reactions of enzyme-bound reduced flavin with oxygen to form C_{4a}-hydroperoxy-FMN. The first phase occurred during dead-time—0.01 s. During this phase, the absorption at 390 nm increased from ~0.04–0.05 AU without any significant change in absorption at 446 nm. A plot of the observed rate constants of this phase (k_{obs1}) versus oxygen concentration (Figure 3b) yielded a calculated bimolecular rate constant (k_{OOH}) of 2.9 × 10^5 M^{-1} s^{-1}. This value is similar to the bimolecular rate constant of the reaction without 4-APA (2.8 × 10^5 M^{-1} s^{-1}; Figure 3a, blue traces; Table 2), indicating that this phase is the reaction of S146A:$FMNH^-$ with oxygen to form the S146A:C_{4a}-hydroperoxy-FMN intermediate (step I in Scheme 1c). The second

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ACS Chem. Biol. XXXX, XXX, XXX–XXX

Table 2. Kinetic Rate Constants of Reactions of Wild-Type and S146A at pH 6.0 and 9.0 According the Scheme 1a−c

reaction	substrate	pH	% yield of 3-OH-4-APA[a]	k_{OOH}[b] (M^{-1} s^{-1})	k_{obs}[c] (s^{-1})	$k_{H_2O_2}$[d] (× 10^{-3} s^{-1})	k_{OH}[e] (× 10^{-3} s^{-1})
wild-type C_2	4-APA	6	83 ± 1	1.1 × 10^6		6	28
		9		1.5 × 10^6		155 (±1)	
wild-type C_2		6		1.1 × 10^6		1.8 (±0.1)	
		9		1.5 × 10^6		159 (±1)	
S146A	4-APA	6	100 ± 1	2.9 × 10^5	26.2, 32.2, 38.6, 41.4		2500 (±200)
		9	82 ± 3	2.5 × 10^5		240	1070
S146A		6		2.9 × 10^5		6.8 (±0.2)	
		9		2.1 × 10^5		28 (±1)	

[a]%Yield of product with 2 mM 4-APA substrate was calculated based on HPLC chromatograms of the formed product and values from a standard 3-OH-4-APA. [b]k_{OOH} = bimolecular rate constant of C_2:$FMNH^-$ reacting with oxygen (step I in Scheme 1a,b,c) [c]k_{obs2} = observed rate constants of S146A:$FMNH^-$:4APA reacting with 0.13, 0.31, 0.61, and 1.03 mM oxygen at pH 6.0 were determined as 26.2, 32.2, 38.6, and 41.4 s^{-1}, respectively (Scheme 1c, step III). [d]$k_{H_2O_2}$ = rate constant of hydrogen peroxide elimination (Scheme 1a, step II; Scheme 1b, step III) [e]k_{OH} = 4-APA hydroxylation rate constant (Scheme 1b, step IV; Scheme 1c, step V)

Table 3. Comparison of Mode of Binding and Hydroxylation Rate Constant (k_{OH}) of Wild-Type and S146A Reactions with 4-HPA and 4-APA at pH 6.0

	wild-type C_2		S146A	
substrate	mode of binding	k_{OH} (s^{-1})	mode of binding	k_{OH} (s^{-1})
4-HPA	binds to C_2:$FMNH^-$ and C_{4a}-hydroperoxyFMN	17[a] (pH 6−10)	binds to C_2:$FMNH^-$ and C_{4a}-hydroperoxyFMN	2.6[b] (pH 7, 9)[c]
4-APA	only binds to C_{4a}-hydroperoxyFMN	0.028 (pH 6.0)	binds to C_2:$FMNH^-$ and C_{4a}-hydroperoxyFMN	2.5 (pH 6.0)

[a]Data are from Ruangchan *et al.*[28] [b]Data are from Tongsook *et al.*[30] [c]The hydroxylation rate constant and product yield of wild-type C_2 with 4-HPA are independent of pH (pH 6.0−10.0).[28] As the product yield of S146A and 4-HPA is also constant throughout a pH range of 6.0−9.0[30] and the hydroxylation rate constants at pH 7.0 and 9.0 are the same, it is likely that the hydroxylation rate constant of S146A and 4-HPA at pH 6.0 is also 2.6 s^{-1}.

phase occurred during 0.01−0.1 s and was oxygen dependent, giving an absorption increase at 390 nm of ∼0.04−0.09 AU with no significant change in absorbance at 446 nm. The observed rate constants (k_{obs2}) of this phase in the presence of 0.13, 0.31, 0.61, and 1.03 mM oxygen were determined as 26.2, 32.2, 38.6, and 41.4 s^{-1}, respectively (Table 2). As at pH 6.0, a ternary complex of S146A:$FMNH^-$:4-APA can be formed before reaction with oxygen (Supporting Information Figure S13a,b), the second phase in Figure 3a is likely a bimolecular reaction of the S146A:$FMNH^-$:4-APA complex with oxygen to form the S146A:C_{4a}-hydroperoxy-FMN:4-APA complex (Scheme 1c, step III). In principle, observed rate constants of this phase should be linearly dependent on oxygen concentration. However, as their observed rate constant values are not well separated from the first phase of reaction, accurate observed rate constants could not be obtained. Therefore, a bimolecular rate constant (k_{OOH}) for the second phase cannot be determined. This reaction is similar to the reaction of wild-type C_2 with 4-HPA,[26,28] in that it has two pathways for oxygen reaction. In the first pathway, S146A:$FMNH^-$ can react with oxygen directly to form S146A:C_{4a}-hydroperoxyFMN (with a bimolecular rate constant, k_{OOH}, of 2.9 × 10^5 M^{-1} s^{-1}) before binding 4-APA to form the S146A:C_{4a}-hydroperoxyFMN:4-APA complex (Scheme 1c, steps I and IV). In the second pathway, S146A:$FMNH^-$ can prebind with 4-APA to form S146A:$FMNH^-$:4-APA and then react with oxygen to generate the ternary complex (Scheme 1c, steps II and III). The third phase occurred at 0.1−1.2 s with an absorption decrease at 390 nm (∼0.015−0.025 AU), corresponding to an observed rate constant (k_{obs3}) of ∼1.5 ± 0.2 s^{-1}. The last phase occurred at 1.2−80 s with a large absorption increase at 446 nm (∼0.28 AU) concomitant with an absorption decrease at 390 nm. The observed rate constant of this phase (k_{obs4} or k_{reox}) was determined as ∼0.048 ± 0.001 s^{-1}. The last two phases were independent of oxygen concentration. These phases involve hydroxylation and the dehydration of C_{4a}-hydroxyFMN to return to the oxidized FMN (Scheme 1c, steps V and VI).

G. Measurement of the Hydroxylation Rate Constant of the Reaction of S146A and 4-APA at pH 6.0 Using Rapid-Quench Flow Techniques. The observed hydroxylation rate constant k_{OH} measured using rapid-quench flow techniques (Figure 3a, red filled circles and red line) indicates that the hydroxylation step occurs during ∼0.05−2 s, essentially concomitant with the third phase of the stopped-flow traces (Figure 3a, black traces), which corresponds to a hydroxylation rate constant of 2.5 ± 0.2 s^{-1} (Figure 3a, red filled circles and red line; Scheme 1c, step V). Because the reaction under these conditions yields ∼100% of product (Table 2), the observed rate constant is thus equal to the actual hydroxylation rate constant (Table 2; Scheme 1c, step V). Interestingly, this hydroxylation rate constant is similar to the hydroxylation rate constant of the reaction of S146A and 4-HPA (Table 3).[30]

Catalytic Feature that Enables S146A to be Efficient in Hydroxylation of 4-APA. All of the transient kinetic results indicate that the wild-type C_2 does not react with 4-APA efficiently (Table 3). In contrast, kinetics of the reactions of S146A with 4-APA and with 4-HPA are similar. Notably, S146A catalyzes hydroxylation of 4-APA and 4-HPA with similar rate constants at pH 6.0 (2.5 s^{-1} versus 2.6 s^{-1}, Table 3). These results indicate that the interaction between Ser146 and the phenolic group of 4-HPA is a key dictating factor in optimizing C_2 reactivity toward 4-HPA. Upon removal of the Ser146 hydroxyl group, the enzyme (S146A) loses its ability to discriminate between 4-APA and 4-HPA and can subsequently catalyze hydroxylation of the unnatural substrate 4-APA with similar reactivity as the natural substrate 4-HPA.

Density Functional Theory Analysis Explains the pH Effect on the Reaction of Wild-Type C_2 and 4-APA.

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ACS Chem. Biol. XXXX, XXX, XXX−XXX

Results in Table 1 and Figure 2 and 3 indicate a higher efficiency in hydroxylation and binding for the wild-type and S146A enzymes at lower pH values. As this effect may be due to the protonation states of 4-APA ($-NH_2$ moiety) and active site residues (such as His120), density functional theory (DFT) was employed to investigate the binding energy of 4-APA to the active site model in the form of C_{4a}-hydroperoxyflavin[35] under three states: (A) protonated 4-APA and neutral His120 (Figure 1b), (B) neutral 4-APA and protonated His120 (Figure 1c), and (C) neutral 4-APA and neutral His120 (Supporting Information Figure S14).

The DFT results indicate that states A and B which contain an extra proton on 4-APA or His120 have binding energies of −54.3 kcal/mol and −47.3 kcal/mol, respectively. These values are greater than those of state C (−37.2 or −39.8 kcal/mol) in which both moieties are neutral. The greater stabilization in states A and B is mainly due to the presence of stronger hydrogen bond interactions than in state C (Figure S14). States A and B likely represent the protonation state of the C_2:4-APA complex at pH 6.0, while state C likely represents the protonation state at pH 9.0. Therefore, the DFT results suggest that at lower pH values, the binding energy of 4-APA is more favorable, allowing the reaction to proceed with higher efficiency under these conditions.

Conclusion. In conclusion, our work has demonstrated that it is possible to expand the reactivity of a two-component flavin-monooxygenase to go beyond the natural substrate. The variant S146A can catalyze the hydroxylation of an aniline substrate with similar reactivity as the natural phenolic substrate. The knowledge acquired by these studies may be applied for future enzyme engineering of C_2 homologues (implications proposed in the Supporting Information).

METHODS

All methods used in this research are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00402.

Methods; HPLC-chromatograms of single turnover reactions of 4-APA reactions; identification of product from single turnover reaction of 4-APA by comparison with a standard 3-OH-4-APA; multiple turnover reactions of wild-type C_2 and S146A with 4-APA at pH 6.0; ^1H and ^{13}C NMR spectra of products from multiple turnover reactions; reactions of wild-type C_2 or S146A:$FMNH^-$ with oxygen in the absence of substrate at pH 6.0 and pH 9.0 monitored by stopped-flow spectrophotometry; reactions of wild-type C_2 and S146A reactions in the presence of 4-APA at pH 9.0 observed by stopped-flow spectrophotometry and rapid-quench technique (PDF)

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Notes

The authors declare no competing financial interest.

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ACS Chem. Biol. XXXX, XXX, XXX−XXX