# Kinetic Isotope Effects on Aromatic and Benzylic Hydroxylation by *Chromobacterium violaceum* Phenylalanine Hydroxylase as Probes of Chemical Mechanism and Reactivity[†]

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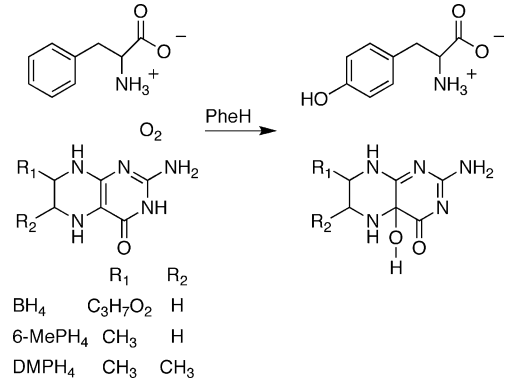
ABSTRACT: Phenylalanine hydroxylase from *Chromobacterium violaceum* (CvPheH) is a non-heme iron monooxygenase that catalyzes the hydroxylation of phenylalanine to tyrosine. In this study, we used deuterium kinetic isotope effects to probe the chemical mechanisms of aromatic and benzylic hydroxylation to compare the reactivities of bacterial and eukaryotic aromatic amino acid hydroxylases. The $^{D}k_{cat}$ value for the reaction of CvPheH with [$^{2}H_5$]phenylalanine is 1.2 with 6-methyltetrahydropterin and 1.4 with 6,7-dimethyltetrahydropterin. With the mutant enzyme I234D, the $^{D}k_{cat}$ value decreases to 0.9 with the latter pterin; this is likely to be the intrinsic effect for addition of oxygen to the amino acid. The isotope effect on the subsequent tautomerization of a dienone intermediate was determined to be 5.1 by measuring the retention of deuterium in tyrosine produced from partially deuterated phenylalanine; this large isotope effect is responsible for the normal effect on $k_{cat}$. The isotope effect for hydroxylation of the methyl group of 4-$CH_3$-phenylalanine, obtained from the partitioning of benzylic and aromatic hydroxylation products, is 10. The temperature dependence of this isotope effect establishes the contribution of hydrogen tunneling to benzylic hydroxylation by this enzyme. The results presented here provide evidence that the reactivities of the prokaryotic and eukaryotic hydroxylases are similar and further define the reactivity of the iron center for the family of aromatic amino acid hydroxylases.

Phenylalanine hydroxylase (PheH)[1] is a non-heme iron monooxygenase that catalyzes the hydroxylation of phenylalanine to form tyrosine (Scheme 1) (*1*). In humans, the enzyme is responsible for catabolism of excess phenylalanine in the diet, and mutations in PheH result in the metabolic disorder phenylketonuria (*2*). In addition, more than 150 bacterial genomes have been reported to include a gene for PheH. The phenylalanine hydroxylase from *Chromobacterium violaceum* (CvPheH) has been the most studied, having been cloned and expressed in *Escherichia coli* (*3, 4*). Scheme 2 shows our current understanding of the mechanism of aromatic amino acid hydroxylation based on studies of the eukaryotic PheH and the other two aromatic amino acid hydroxylases, tyrosine hydroxylase (TyrH) and tryptophan hydroxylase (*5*). After the three substrates are bound, molecular oxygen forms a peroxo bridge between the 4a position of the pterin and the iron. The oxygen−oxygen bond

Scheme 1




then cleaves to form the Fe(IV)O hydroxylating species and the 4a-hydroxypterin product (*6*−*8*). Direct evidence for Fe(IV)O as the hydroxylating intermediate has recently been obtained for TyrH (*9*). Once formed, the Fe(IV)O reacts with the side chain of the amino acid by electrophilic aromatic substitution (*10*), generating a carbocation that undergoes a 1,2-hydride transfer to form a dienone. Tautomerization of the latter gives the final tyrosine product.

The sequence of CvPheH is ~35% identical with the corresponding residues of human PheH, and its X-ray crystal structure can be superimposed on the catalytic domain of the human enzyme with a rmsd of 1.2 Å (*11*). The homology between bacterial and eukaryotic PheH extends to the catalytic domains of TyrH and tryptophan hydroxylase (*12, 13*).

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[1] Abbreviations: PheH, phenylalanine hydroxylase; CvPheH, *C. violaceum* phenylalanine hydroxylase; TyrH, tyrosine hydroxylase; 6-$MePH_4$, 6-methyltetrahydropterin; $DMPH_4$, 6,7-dimethyltetrahydropterin; $BH_4$, tetrahydrobiopterin; DTT, dithiothreitol; rmsd, root-mean-square deviation.

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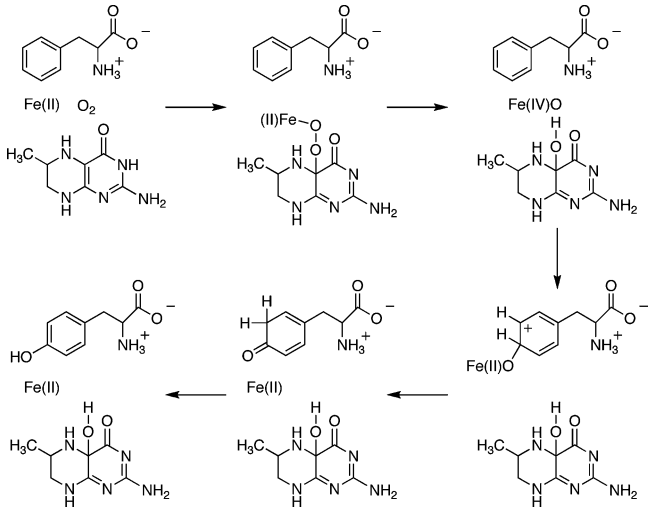
Scheme 2




Despite these structural similarities, a number of lines of evidence have suggested that there are significant differences between the eukaryotic and bacterial enzymes. On the basis of the differences in the structures and kinetics between the eukaryotic and bacterial PheHs, Erlandsen et al. (*11*) have proposed that the bacterial enzyme is more optimized for phenylalanine hydroxylation, implying a more reactive hydroxylating species. While eukaryotic TyrH and PheH both require iron for catalysis of tetrahydropterin oxidation (*14*−*16*), Chen and Frey (*4*) have reported that iron-free CvPheH catalyzes the oxidation of 6,7-dimethyltetrahydropterin ($DMPH_4$) at ∼5% the rate of the iron-containing enzyme, suggesting that iron is not necessary for the initial steps in oxygen activation by this enzyme. Finally, while it is well-accepted that the three eukaryotic aromatic amino acid hydroxylases are non-heme iron enzymes (*17*−*19*), the affinity of CvPheH for various metals, including iron, has resulted in some uncertainty in the literature regarding its metal requirement. The enzyme was originally described as copper-dependent (*20*), then as metal-independent (*21*), and more recently as iron-dependent (*4*).

The goal of this work was to use kinetic isotope effects as probes of transition-state structures for hydroxylation reactions catalyzed by CvPheH to allow comparison with the reactivities of the eukaryotic enzymes. In addition to aromatic hydroxylation, the bacterial and eukaryotic PheHs are capable of hydroxylating benzylic carbons (*21, 22*). In the case of the eukaryotic enzymes, the deuterium kinetic isotope effect on benzylic hydroxylation is consistent with a mechanism involving abstraction of a hydrogen atom from the methyl group (*23*); this reaction has previously been used to compare the reactivities of the Fe(IV)O intermediates in the three eukaryotic enzymes (*23*).

## EXPERIMENTAL PROCEDURES

*Materials.* Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Plasmid pET21b was from Novagen (San Diego, CA). Restriction and DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA) and Promega (Madison, WI). Plasmids were purified using Wizard mini-prep kits from Promega. Catalase was from Roche (Indianapolis, IN). DEAE-Sephacel was from Amersham Pharmacia Biotech (Uppsala, Sweden). *E. coli* strain BL21(DE3), used for protein expression, was from Novagen, and strain Mach1, used for subcloning, was from Invitrogen (Carlsbad, CA). 6-Methyltetrahydropterin (6-$MePH_4$), tetrahydrobiopterin ($BH_4$), and $DMPH_4$ were from B. Schircks Laboratories (Jona, Switzerland). L-Tyrosine, L-phenylalanine, L-tryptophan, D,L-phenylalanine, dihydropteridine reductase, sodium cyanide, boric acid, and 5-hydroxytryptophan were from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Naphthalene 2,3-dicarboxaldehyde was from Invitrogen. L-[ring-$^2H_5$]Phenylalanine was from Cambridge Isotope Laboratories (Andover, MA). Dithiothreitol (DTT) was from Inalc (Milan, Italy). The syntheses of D,L-[4-$^2$H]phenylalanine, D,L-[3,5-$^2H_2$]phenylalanine, and 4-$C^2H_3$-phenylalanine were described previously (*24, 25*). All other reagents were of the highest purity commercially available.

*Construction of Vectors, Enzyme Expression, and Purification.* A vector containing the gene for *C. violaceum* phenylalanine hydroxylase was obtained from P. Frey (University of Wisconsin, Madison, WI). The DNA was amplified by PCR and subcloned into the pET21b vector using the 5′ NdeI and 3′ HindIII restriction sites to generate the plasmid pET21b-CvPheH. The I234D mutation was introduced into pET21b-CvPheH using the QuikChange protocol (Stratagene). Both plasmids were sequenced by the Gene Technology Laboratory of the Department of Biology of Texas A&M University.

Purification of wild-type and I234D CvPheH was carried out with modifications of previously reported procedures (*3, 11*). *E. coli* strain BL21(DE3) was transformed with plasmid pET21b-CvPheH. A single colony was used to inoculate 50 mL of LB broth (100 $\mu$g/mL ampicillin) and allowed to grow at 37 °C for 16 h. Ten milliliters of the overnight culture was used to inoculate 1 L of fresh LB broth (100 $\mu$g/mL ampicillin). When the $A_{600}$ reached a value between 0.8 and 1.0, the cells were induced with isopropyl $\beta$-thiogalactoside at a final concentration of 120 mg/L. After 5 h at 37 °C, the cells were harvested by centrifugation for 30 min at 2600*g*. The cell pellet was suspended in an 8-fold excess (with respect to the initial weight of the cells) of 50 mM Hepes buffer (pH 7.2), 1 mM DTT, and 100 $\mu$g/mL phenylmethanesulfonyl fluoride (dissolved in acetone). Cells were disrupted using 100 mg/mL lysozyme and five 3 min sonication cycles. The suspension was centrifuged at 17600*g* for 30 min. The supernatant was made 600 $\mu$M in $FeSO_4$ and stirred for 30 min at 4 °C. The solution was brought to 2% streptomycin sulfate, stirred for 15 min, and centrifuged for 30 min at 17600*g*. The resulting solution was made 50% saturated in ammonium sulfate, stirred for 15 min, and centrifuged for 30 min at 17600*g*. The resulting protein pellet was dissolved in a minimal amount of 50 mM Hepes (pH 7.2) and 20 mM NaCl and dialyzed for 4 h against a 100-fold excess of the same buffer solution with two buffer changes. The dialyzed solution was loaded onto a DEAE-Sephacel column equilibrated with 50 mM Hepes (pH 7.2) and 20 mM NaCl. The protein was eluted with a linear gradient formed with 500 mL of 50 mM Hepes buffer (pH 7.2) containing 20 mM NaCl and 500 mL of the same buffer containing 500 mM NaCl. Fractions containing the purest

and most active enzyme were pooled. This method usually gave 100 mg of more than 95% pure CvPheH per liter of LB.

Metal-depleted CvPheH was obtained by incubating the enzyme with 2 mM EDTA followed by dialysis against 50 mM Hepes (pH 7.2) and 20 mM NaCl. For the $DMPH_4$ oxidation studies, gel filtration chromatography using Micro Bio-Spin columns from Bio-Rad (Hercules, CA) was carried out to remove the EDTA. The metal content of the protein was measured as previously described (*26*) using a Perkin-Elmer model 2380 atomic absorption spectrophotometer equipped with a graphite furnace.

*Enzyme Assays.* All spectrophotometric assays were carried out in a Hewlett-Packard model 8453 diode array spectrophotometer equipped with a thermostatically controlled cuvette holder. Tyrosine formation was assessed by monitoring the change in absorbance at 275 nm (*27, 28*); an $\varepsilon_{275}$ value of 1.34 $mM^{-1}$ $cm^{-1}$ was used to calculate the rate of product formation. The assays were performed at 25 °C with 50 mM Hepes (pH 7.2), 5 mM DTT, 10 $\mu$M ferrous ammonium sulfate, 50 $\mu$g/mL catalase, 0.1−0.3 $\mu$M CvPheH, 150 $\mu$M 6-$MePH_4$ or 250 $\mu$M $DMPH_4$, and varying concentrations of phenylalanine. The steady-state kinetic parameters for the hydroxylation of tryptophan were measured using a coupled assay in which the oxidation of NADH by dihydropteridine reductase was followed (*28*).

The coupling between amino acid oxidation and tetrahydropterin oxidation was assessed using a HPLC-based assay as previously described (*29*). The conditions included 500 $\mu$M phenylalanine, 10 $\mu$M ferrous ammonium sulfate, 1 $\mu$M CvPheH, and 25−100 $\mu$M tetrahydropterin. The reactions were carried out for 5 min in 400 $\mu$L of 10 mM sodium phosphate buffer (pH 7.0). The reaction was quenched with 100 $\mu$L of 100 mM sodium borate (pH 9.0). To this were added 50 $\mu$L of 50 mM sodium cyanide and 100 $\mu$L of 50 mM naphthalene-2,3-dicarboxaldehyde. The fluorescent derivatives were separated using a Nova-Pack C18 column and a gradient from 30 to 40% acetonitrile in 10 mM sodium phosphate (pH 7.0) with 1% THF. The fluorescent molecules were detected using a Waters 2475 detector. The excitation and emission wavelengths were 420 and 490 nm, respectively. The amount of tyrosine was quantified using a standard curve generated using the same reaction conditions but without the protein.

The products from the reaction of CvPheH with 4-$CH_3$-phenylalanine were detected and quantified using a HPLC-based assay as previously described (*25*). The conditions for the reaction included 500 $\mu$M 4-$CH_3$-phenylalanine or 4-$C^2H_3$-phenylalanine, 10 $\mu$M ferrous ammonium sulfate, 1 $\mu$M CvPheH, and 250 $\mu$M tetrahydropterin. The reactions were carried out for 2 min in 300 $\mu$L of 10 mM sodium phosphate buffer (pH 7.0). Samples for determination of the isotopic content of the hydroxylated amino acid products were prepared by HPLC following essentially the same protocol; 1 mM 6-$MePH_4$ or $DMPH_4$, 1 $\mu$M CvPheH, and 500 $\mu$M D,L-[4-$^2$H]phenylalanine or D,L-[3,5-$^2H_2$]phenylalanine were used in the reactions. The reactions were performed at 25 °C for 30 min. The peak corresponding to tyrosine was collected and analyzed by negative ion electrospray time-of-flight mass spectrometry at the Laboratory of Biological Mass Spectrometry at Texas A&M University.

Table 1: Effect of Storage Conditions on the Activity of CvPheH[a]

| enzyme condition | $K_{Phe}$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|
| recently purified | 69 ± 7 | 11 ± 1 |
| after 1 month at −80 °C | 105 ± 8 | 7.9 ± 0.2 |
| after 1 month at −80 °C treated with 2 mM EDTA | 68 ± 11 | 12 ± 1 |

[a] The solution contained 50 mM HEPES (pH 7.2), 5 mM DTT, 10 $\mu$M ferrous ammonium sulfate, 50 $\mu$g/mL catalase, 150 $\mu$M 6-$MePH_4$, and 0.1−0.3 $\mu$M CvPheH.

The data were corrected for $^{13}$C contributions and used in the calculation of the isotope effects.

*Data Analysis.* Initial rates obtained as a function of the concentration of a single substrate were fit to the Michaelis−Menten equation to yield $k_{cat}$, $k_{cat}/K_M$, and $K_M$ values using KaleidaGraph (Synergy Software, Reading, PA). When substrate inhibition was observed, the data were fit to eq 1, in which $K_i$ is the substrate inhibition constant. When the DTT concentration was varied, the data were fit to eq 2. Here, $v_0$ and $v_\infty$ are the rates when DTT is absent and at a saturating level, respectively. Steady-state kinetic isotope effects were determined using Igor Pro (WaveMetrics, Lake Oswego, OR) to fit the data to eq 3 which assumes an isotope effect on $k_{cat}$ only. Here, $v$ is the initial rate, $e$ the enzyme concentration, $F_i$ the fraction of deuterium in the substrate, and $E_v$ the isotope effect on $k_{cat}$.

$$v/e = k_{cat}S/(K_M + S + S^2/K_i) \quad (1)$$

$$v = v_0 + v_\infty A/(K_A + A) \quad (2)$$

$$v/e = k_{cat}S/\{K_M + S[1 + F_i(E_v - 1)]\} \quad (3)$$

## RESULTS

*Activity of CvPheH.* Because of contradictory reports in the literature, we re-examined the metal content and kinetics of CvPheH. In our hands, the recombinant enzyme expressed in *E. coli* contained significant amounts of copper, zinc, and iron when isolated. The activity of this enzyme was only 30% of the reported value (*4, 11*) and was correlated with the amount of iron present in the enzyme. Fully active, 100% iron-containing enzyme could be obtained by addition of $FeSO_4$ to the cell extract. However, we observed a decrease in activity of the enzyme purified in this fashion after prolonged periods at −80 °C. The activity of the protein could be restored after treatment with 2 mM EDTA and subsequent addition of ferrous ammonium sulfate after dialysis, suggesting a slow inhibition by other metals during the storage period (Table 1).

It was previously reported that CvPheH requires DTT to couple tetrahydropterin oxidation to the hydroxylation of phenylalanine and that no tyrosine is formed without the addition of a thiol (*4*). However, we found that in the absence of DTT the activity of recently purified CvPheH is 50% of that achieved when DTT is present. The effect of the concentration of DTT on the activity is shown in Figure 1. Fitting the data to eq 2 yields a value of 0.6 ± 0.2 mM for the concentration of DTT giving half-maximal activation. Accordingly, DTT was routinely included in assays.

The steady-state parameters for the fully active enzyme were consistent with previous reports (*21, 30, 31*), with the following order of preference for the tetrahydropterin substrates: $DMPH_4$ > 6-$MePH_4$ > $BH_4$. While we also found

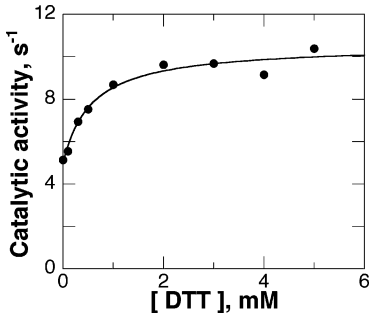



FIGURE 1: Initial rates of tyrosine formation by phenylalanine hydroxylase as a function of DTT concentration. The line is from the fit of the data to eq 2.

Table 2: Kinetic Isotope Effects on $k_{cat}$ Values for CvPheH with Deuterated Phenylalanine[a]

| enzyme | 6-MePH$_4$ | DMPH$_4$ |
|---|---|---|
| wild type | 1.24 ± 0.04 | 1.43 ± 0.07 |
| I234D | 1.00 ± 0.01 | 0.90 ± 0.03 |

[a] Conditions as described in footnote a of Table 1.

that tyrosine is not a substrate, in our hands CvPheH has substantial activity with tryptophan [$k_{cat(trp)} = 2.2 \pm 0.2$ s$^{-1}$ and $k_{cat}/K_{trp} = 3 \pm 1$ s$^{-1}$ mM$^{-1}$ vs $k_{cat(phe)} = 12 \pm 1$ s$^{-1}$ and $k_{cat}/K_{phe} = 180 \pm 20$ s$^{-1}$ mM$^{-1}$], in contrast to previous reports of very low activity with this amino acid (*30*).

*Isotope Effects on Aromatic Hydroxylation.* The mechanism of Scheme 2 predicts that multiple steps will be affected by deuterium substitution of the amino acid substrate at the site of hydroxylation. Deuterium kinetic isotope effects on the rate of tyrosine formation were determined for CvPheH with [ring-$^2H_5$]phenylalanine. The best fit of the data was to eq 3, which assumes an isotope effect on only $k_{cat}$. The observed kinetic isotope effect was normal with both 6-MePH$_4$ and DMPH$_4$ (Table 2). A similar result has been reported for rat PheH (*29, 32*). With that enzyme, the normal isotope effect is a combination of two isotope-sensitive steps, the initial reaction of the hydroxylating intermediate with the aromatic ring of phenylalanine and the final tautomerization to form tyrosine (*29*).[2]

For rat PheH and TyrH, the intrinsic isotope effect on the initial formation of the carbon−oxygen bond could be obtained using mutant enzymes in which an alternative pathway for the decay of the hydroxylating intermediate was introduced (*29, 34*). In the case of rat PheH, the single V379D mutation was sufficient to unmask the intrinsic isotope effect on hydroxylation. Consequently, we introduced the corresponding mutation, I234D, into CvPheH. The hydroxylation of phenylalanine by CvPheH I234D was only 60 and 30% coupled to the oxidation of 6-MePH$_4$ and DMPH$_4$, respectively. Moreover, the isotope effect observed with DMPH$_4$ was inverted with the mutant enzyme, and the isotope effect observed with the more coupled 6-MePH$_4$ was clearly less normal than for the wild-type enzyme (Table 2). The value of 0.90 obtained with the mutant protein and DMPH$_4$ is within error of the intrinsic kinetic isotope effect for addition of oxygen to the aromatic ring of the amino acid substrate by both TyrH (*34*) and rat PheH (*29*). It is thus likely to be the intrinsic isotope effect for that step with CvPheH also.

Table 3: Ratios of Deuterium to Protium in the Tyrosine Produced by CvPheH[a]

| tetrahydropterin | [4-$^2$H]Phe | [3,5-$^2H_2$]Phe |
|---|---|---|
| 6-MePH$_4$ | 6.3 ± 0.5 | 4.1 ± 0.2 |
| DMPH$_4$ | 5.7 ± 0.3 | 4.4 ± 0.3 |

[a] Conditions: 10 mM phosphate buffer (pH 7.0), 10 μM ferrous ammonium sulfate, 1 μM CvPheH, 400 μM phenylalanine, and 3 mM DMPH$_4$ or 6-MePH$_4$. After 30 min, the reaction was stopped, the amino acid products were purified by HPLC, and their deuterium content was determined using ESI mass spectrometry.

To measure the isotope effect on the subsequent tautomerization of the dienone to tyrosine (Scheme 2), the deuterium content of tyrosine produced from [4-$^2$H]- or [3,5-$^2H_2$]phenylalanine was determined using mass spectrometry. While hydrogen is preferentially lost in all cases, there is slightly more deuterium in the tyrosine from [4-$^2$H]phenylalanine, independent of the identity of the pterin (Table 3). The kinetic isotope effect on the tautomerization can be determined from the data listed in Table 3 using the minimal mechanism in Scheme 3 (*29*). After the 1,2-hydride shift, both atoms are on the carbon adjacent to the site of hydroxylation. To form tyrosine, $H_a$ can be lost with rate constant $k_a$ or $H_b$ with rate constant $k_b$. When [4-$^2$H]phenylalanine is the substrate, $k_a$ is subject to a primary deuterium isotope effect. Likewise, $k_b$ is subject to an isotope effect when [3,5-$^2H_2$]phenylalanine is the substrate. The isotope effects on $k_a$ and $k_b$ are related to the deuterium content of the tyrosine products from [4-$^2$H]- and [3,5-$^2H_2$]phenylalanine by eqs 4 and 5, respectively. Here, $P_D$ and $P_H$ (eq 4) are the relative amounts of tyrosine retaining one and zero deuterium atoms in the reaction with [4-$^2$H]phenylalanine, respectively, while $P_{D2}$ and $P_D$ in eq 5 are the relative amounts of tyrosine containing two and one deuterium atoms in the reaction with [3,5-$^2H_2$]phenylalanine, respectively. If $k_a$ and $k_b$ are affected in the same way by deuterium substitution, then the geometric mean of the ratios reported in Table 3 gives the isotope effect for the tautomerization (*35*). This value is 5.1 ± 1.0 for the reactions with both 6-MePH$_4$ and DMPH$_4$.

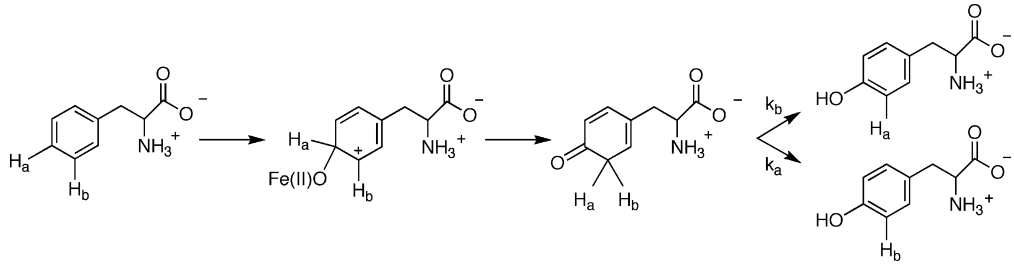
$$P_D/P_H = R_1 = {}^Dk_a(k_b/k_a) \quad (4)$$

$$P_{D2}/P_D = R_2 = {}^Dk_b(k_a/k_b) \quad (5)$$

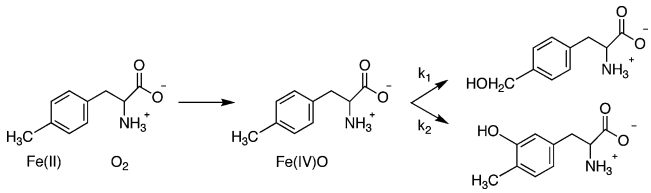
*Isotope Effects on Benzylic Hydroxylation.* The eukaryotic and prokaryotic hydroxylases will catalyze hydroxylation on the benzylic carbon as well as on the aromatic ring of 4-methylphenylalanine (Scheme 4) (*21, 23, 25*). In the case of CvPheH, we found that 89% of the product is 4-HOCH$_2$-phenylalanine and 11% 4-HO-3-CH$_3$-phenylalanine. However, when 4-C$^2$H$_3$-phenylalanine is the substrate, 45% of the product comes from benzylic hydroxylation and 55% from aromatic hydroxylation. The intrinsic isotope effect on benzylic hydroxylation, ${}^Dk_{Benz}$, is related to the isotope effect on the fraction of 4-HOCH$_2$-phenylalanine produced through eq 6 (*25, 35*). Here, $k_1$ and $k_2$ are the net rate constants for the reaction of the hydroxylating intermediate with the nondeuterated substrate for benzylic and aromatic hydroxylation, respectively (Scheme 4). Application of eq 6 to the data for CvPheH yields an intrinsic isotope effect on benzylic

[2] While the intervening step in Scheme 2, the 1,2-hydride shift, involves cleavage of a carbon−hydrogen bond, computations predict that this step will have a low energy barrier and be isotope-insensitive (*33*).

Scheme 3



Scheme 4



hydroxylation of 10 ± 1 at 25 °C. This large isotope effect suggests a contribution of hydrogen tunneling to catalysis. To test this possibility, we measured the temperature dependence of the isotope effect on benzylic hydroxylation (Figure 2). The isotope effects at different temperatures were fit to eq 7 to yield an isotope effect on the Arrhenius prefactor ($A_H/A_D$) of 0.29 ± 0.03 and a difference in activation energy ($\Delta E_a$) for hydrogen and deuterium of 2.1 ± 0.4. These results show a moderate contribution of tunneling to the benzylic hydroxylation reaction catalyzed by CvPheH (*36*).

$$^{D}(\text{fraction of benzylic hydroxylation}) = ({}^{D}k_{Benz} + k_f/k_2)/(1 + k_f/k_2) \quad (6)$$

$$\ln(k_H/k_D) = \ln(A_H/A_D) + [E_a(D) - E_a(H)]/RT \quad (7)$$

*Uncoupled DMPH₄ Oxidase Activity of CvPheH.* Chen and Frey (*4*) reported that metal-free CvPheH is able to catalyze the phenylalanine-dependent oxidation of $DMPH_4$ at ~5% of the rate of the metal-containing enzyme. This result was interpreted as support of a model in which the initial reaction of molecular oxygen is with the tetrahydropterin with no need for the iron. To confirm this result, we incubated CvPheH with EDTA to remove all the bound metal. After dialysis to remove the EDTA, analysis of the metal content of this apo-CvPheH showed that iron was present at less than 1 mol %. In the absence of added iron in the assay, the EDTA-treated CvPheH still exhibited 3.5% of the phenylalanine hydroxylation activity of the metal-containing enzyme, consistent with the results of Chen and Frey (*4*). However, in our hands, tyrosine formation and $DMPH_4$ oxidation were completely coupled, and increasing amounts of enzyme yielded decreasing $k_{cat}$ values, suggesting that the residual activity was due to a very low concentration of iron in the dialysis buffers or assay. To avoid exposure of the apoenzyme to adventitious iron during dialysis to remove excess EDTA, the chelator was removed by gel filtration. In addition, we included 10 μM EDTA in the assays to scavenge any free metal. When CvPheH was treated in this fashion, the level of formation of tyrosine was reduced below the detection limit of 8 nM, and the rate of $DMPH_4$ oxidation was unaffected by the presence of the enzyme (Table 4).

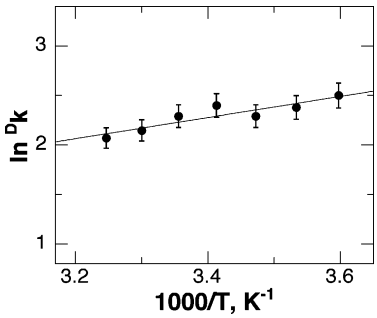



FIGURE 2: Temperature dependence of the isotope effect on benzylic hydroxylation for the reaction of phenylalanine hydroxylase and 4-methylphenylalanine. Each point is the average of three experiments.

Table 4: Effect of CvPheH on the Rate of $DMPH_4$ Oxidation[a]

| CvPheH | rate (nmol $s^{-1}$) |
|---|---|
| no CvPheH | 0.034 ± 0.002 |
| EDTA-treated CvPheH (0.1 μM) | 0.030 ± 0.002 |
| EDTA-treated CvPheH (0.1 μM) with Fe(II) (15 μM) | 1.2 ± 0.01 |

[a] Conditions: 100 mM HEPES (pH 7.2), 3 mM phenylalanine, 150 μM $DMPH_4$, and 10 μM EDTA. The rate was determined from the change in $A_{437}$ with time using an $\varepsilon_{437}$ value of 3600 $M^{-1}$ $cm^{-1}$.

## DISCUSSION

Several authors (*37, 38*) have proposed that the eukaryotic amino acid hydroxylases evolved from a common ancient hydroxylase which resembled the bacterial phenylalanine hydroxylase. Consistent with such a model, bacterial and eukaryotic phenylalanine hydroxylases have 35% identical sequences and similar structures for their catalytic domains (*11*−*13, 39*). While this makes the bacterial enzyme an attractive and simpler model for the study of the mechanism of aromatic amino acid hydroxylation, structural differences and the different metal binding ability of the bacterial enzyme raise the possibility that there are substantive differences in the reactivities of the bacterial and eukaryotic enzymes. Comparison of the intrinsic isotope effects for the bacterial enzyme with previously reported values for the eukaryotic enzyme provides a test of this hypothesis, in that the intrinsic isotope effects reflect the transition-state structures for the individual reactions and thus of the reactivity of the Fe(IV)O intermediate in the different enzymes.

The physiological reaction catalyzed by CvPheH, the hydroxylation of phenylalanine to form tyrosine, exhibits a deuterium kinetic isotope effect of >1 with both 6-$MePH_4$ and $DMPH_4$. The value with $DMPH_4$ is similar to that for the catalytic domain of rat PheH (*29*), while that with 6-$MePH_4$ is somewhat smaller. Neither value is the intrinsic isotope effect for the reaction of the Fe(IV)O intermediate with the aromatic ring of the substrate. Rather, the observed

isotope effects are combinations of the isotope effect on this step and the isotope effect on the subsequent tautomerization to form phenylalanine. For the three eukaryotic enzymes, the isotope effects on the initial hydroxylation are all 0.90 ± 0.03 (*7, 29, 34*). With both rat PheH and rat TyrH, this isotope effect is masked by other slower steps and could be measured only in mutant proteins in which an unproductive side path was introduced. This same strategy was effective with CvPhe, in that the I234D enzyme exhibits a $^{D}k_{cat}$ value identical to the intrinsic isotope effects for the eukaryotic enzymes when the less-coupled $DMPH_4$ is used as the substrate. This agreement of the isotope effects and the fact that homologous mutations in CvPheH and rat PheH unmask it are consistent with similar transition states for hydroxylation by both enzymes. The isotope effects for the subsequent tautomerization step are also identical for rat and CvPheH at 5.1, suggesting that this step is also identical in the two enzymes. The much smaller values for the observed isotope effects are consistent with hydroxylation or an isotope-insensitive step being ~9-fold slower than tautomerization. Tautomerization is expected to be much more favorable in the absence of a catalyst than formation of the Fe(IV)O species or the hydroxylation step, raising the possibility that there is no need for the enzyme to actively catalyze this step, so that it occurs after product release. However, with CvPheH, there is a 20% preference for loss of the hydrogen initially present at the 3-position of the aromatic ring. In the case of rat PheH, there is a slight (34%) preference for loss of the other hydrogen (*29*). These results are most consistent with this step occurring in the active site of both enzymes and with the small differences in the structures seen in the X-ray structures.

The magnitude of the isotope effect on benzylic hydroxylation is a far more sensitive probe of the reactivities of the Fe(IV)O intermediate in the prokaryotic and eukaryotic enzymes due to its much greater magnitude. The relative amount of benzylic hydroxylation of 4-methylphenylalanine is greater with CvPheH (89%) than with the rat enzyme (55%) (*23*), further establishing that the active sites are very similar but not identical. The isotope effect for benzylic hydroxylation of 10 ± 1 is slightly smaller than the value for the catalytic domain of the rat enzyme, 12 ± 1, but this is probably not significant. Importantly, benzylic hydroxylation by CvPheH also involves moderate tunneling of the substrate hydrogen atom. Of the two parameters derived from the temperature dependence of the isotope effect on this reaction, the effect of deuteration on the activation energy, $\Delta E_a$, can be measured with greater confidence than the effect on the Arrhenius prefactor, in that the latter involves extrapolation to infinite temperature. The former value for CvPheH is within error of the value for rat PheH. The interpretation of the temperature dependence of kinetic isotope effects is a matter of intense investigation at present, and no final consensus has been reached. While temperature-dependent isotope effects such as those described here for benzylic hydroxylation by CvPheH can be rationalized using a simple correction to the transition state such that hydrogen tunnels through the barrier at a lower energy than deuterium (*40*), the temperature dependence of the isotope effects for a growing number of enzymes cannot be explained by such a simple model (*41*−*44*). Instead, more sophisticated models are required in which the temperature dependence reflects the extent of preorganization of the active site and/or the involvement of protein motion in the reaction coordinate (*45*−*48*). In these models, the significant temperature dependence of the isotope effect for benzylic hydroxylation would reflect an active site which is not optimized for the reaction. Such a model is certainly reasonable in that PheH and the other aromatic amino acid hydroxylases have not been designed by evolution for this reaction. More importantly, the similar effects of temperature on the isotope effects for CvPheH and the eukaryotic enzymes indicate similar active site environments for hydroxylation in all.

Chen and Frey (*4*) reported that iron-free CvPheH was able to catalyze the oxidation of $DMPH_4$ without the formation of tyrosine. However, the results in Table 4 show that this activity is lost when metal-free CvPheH is used. These two results can be reconciled if the residual tetrahydropterin oxidation activity reported previously is conferred by a metal other than iron. A precedent for this is the H336Q mutant of tyrosine hydroxylase, which in the presence of Co(II) catalyzes the oxidation of tetrahydropterin without hydroxylation of tyrosine (*16*).

The isotope effects reported here for hydroxylation of phenylalanine and 4-methylphenylalanine by CvPheH, the temperature dependence of the latter, and the iron requirement for catalysis of tetrahydropterin oxidation by CvPheH all demonstrate that the reactivity of the hydroxylating intermediate in this bacterial amino acid hydroxylase is indistinguishable from that of the eukaryotic enzyme. Thus, for mechanistic if not regulatory studies, the bacterial enzyme is a valid model for the eukaryotic enzymes.

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