Insights into the Catalytic Mechanisms of Phenylalanine and Tryptophan Hydroxylase from Kinetic Isotope Effects on Aromatic Hydroxylation†

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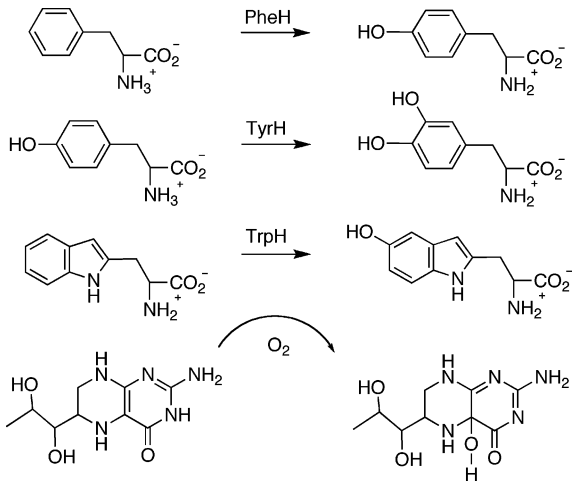
ABSTRACT: Phenylalanine hydroxylase (PheH) and tryptophan hydroxylase (TrpH) catalyze the aromatic hydroxylation of phenylalanine and tryptophan, forming tyrosine and 5-hydroxytryptophan, respectively. The reactions of PheH and TrpH have been investigated with [4-^{2}H]-, [3,5-$^2H_2$]-, and $^2H_5$-phenylalanine as substrates. All $^Dk_{cat}$ values are normal with Δ117PheH, the catalytic core of rat phenylalanine hydroxylase, ranging from 1.12−1.41. In contrast, for Δ117PheH V379D, a mutant protein in which the stoichiometry between tetrahydropterin oxidation and amino acid hydroxylation is altered, the $^Dk_{cat}$ value with [4-^{2}H]-phenylalanine is 0.92 but is normal with [3,5-$^2H_2$]-phenylalanine. The ratio of tetrahydropterin oxidation to amino acid hydroxylation for Δ117PheH V379D shows a similar inverse isotope effect with [4-^{2}H]-phenylalanine. Intramolecular isotope effects, determined from the deuterium contents of the tyrosine formed from [4-^{2}H]-and [3,5$^2H_2$]-phenylalanine, are identical for Δ117PheH and Δ117PheH V379D, suggesting that steps subsequent to oxygen addition are unaffected in the mutant protein. The inverse effects are consistent with the reaction of an activated ferryl-oxo species at the para position of the side chain of the amino acid to form a cationic intermediate. The normal effects on the $^Dk_{cat}$ value for the wild-type enzyme are attributed to an isotope effect of 5.1 on the tautomerization of a dienone intermediate to tyrosine with a rate constant 6- to7-fold that for hydroxylation. In addition, there is a slight (∼34%) preference for the loss of the hydrogen originally at C4 of phenylalanine. With $^2H_5$-indole-tryptophan as a substrate for Δ117PheH, the $^Dk_{cat}$ value is 0.89, consistent with hydroxylation being rate-limiting in this case. When deuterated phenylalanines are used as substrates for TrpH, the $^Dk_{cat}$ values are within error of those for Δ117PheH V379D. Overall, these results are consistent with the aromatic amino acid hydroxylases all sharing the same chemical mechanism, but with the isotope effect for hydroxylation by PheH being masked by tautomerization of an enedione intermediate to tyrosine.

Phenylalanine hydroxylase (PheH[1]), tyrosine hydroxylase (TyrH), and tryptophan hydroxylase (TrpH) form a small family of nonheme iron monooxygenases (*1*). These enzymes catalyze the insertion of an oxygen atom from molecular oxygen into the aromatic side chain of their corresponding substrates, utilizing a tetrahydropterin to reduce the other oxygen atom to the level of water (Scheme 1) (*2*). The three aromatic amino acid hydroxylases show significant sequence and structural similarity, especially in their catalytic cores. Structures are now available for the isolated catalytic domains of all three enzymes, clearly showing the structural homology (*3*−*5*). This homology extends to the active site where each contains an iron atom coordinated by a glutamate and two histidine residues. This arrangement of iron ligands has also been found in a number of other nonheme iron mono and dioxygenases and has been termed the 2-His-1-carboxylate facial triad (*6*, *7*). The chemical mechanism proposed for the aromatic amino acid hydroxylases is shown in Scheme 2 (*2*). After all the substrates are bound, tetrahydropterin, oxygen, and the iron react to form a high valence ferryl-oxo

Scheme 1



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[1] Abbreviations: TyrH, tyrosine hydroxylase; PheH, rat phenylalanine hydroxylase; TrpH, tryptophan hydroxylase; Δ117PheH, the catalytic core of phenylalanine hydroxylase lacking 117 residues from the *N*-terminus; $TrpH_{102-416}$, the catalytic core of rabbit tryptophan hydroxylase lacking 101 and 28 residues from the amino and carboxyl termini, respectively; 6-$MePH_4$, 6-methyltetrahydropterin; DHPR, dihydropteridine reductase; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethane-sulfonic acid; NDA, naphthalene-2,3-dicarboxaldehyde; CBI, 1-cyanobenz[*f*]-isoindole.

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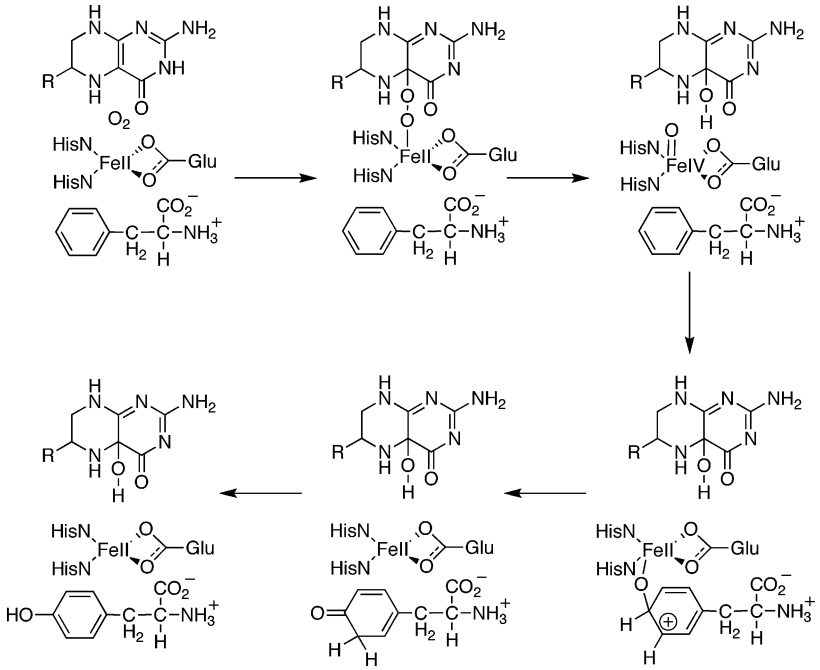
Scheme 2



intermediate and 4a-hydroxypterin. An electrophilic substitution reaction between the ferryl-oxo intermediate and the substrate proceeds through a cationic intermediate that then undergoes a 1,2-hydrogen (NIH) shift and tautomerization to yield the hydroxylated amino acid product and the ferrous enzyme.

In the mechanism of Scheme 2, the rate constant for the formation of the new carbon−oxygen bond upon attack of the ferryl-oxo species should increase 10−15% upon deuterium substitution at the site of hydroxylation, resulting in a $^{D}k_{cat}$ value of about 0.9 if this step is fully rate limiting. In the case of TrpH, such an inverse isotope effect is indeed found (*8*). In contrast, with wild-type TyrH, no isotope effect is found when 3,5-$^{2}H_2$-tyrosine is used as the substrate (*9*). In this case, an isotope effect could be unmasked by taking advantage of mutant enzymes that oxidize excess tetrahydropterin relative to the amount of amino acid hydroxylated (*10*). Several active site mutants of TyrH yield $^{D}k_{cat}$ values similar to the value for TrpH, consistent with these two aromatic amino acid hydroxylases sharing the same chemical mechanism. In the case of wild-type PheH, a normal kinetic isotope effect on k_{cat} of 1.45 has been reported (*11*), suggesting a different rate-limiting step for PheH or perhaps a different chemical mechanism than that of the other two hydroxylases. In the present study, an approach similar to that taken for TyrH was carried out with Δ117PheH, employing mutant enzymes and kinetic isotope effects to probe individual steps in the catalytic mechanism.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer by the Gene Technology Laboratory of the Biology Department of Texas A&M University. 6-Methyltetrahydropterin (6-MePH$_4$) was from B. Schircks Laboratories (Jona, Switzerland). L-Tyrosine, L-phenylalanine, L-tryptophan, D,L-phenylalanine, sheep dihydropteridine reductase (DHPR), NADH, sodium cyanide, boric acid, and 5-hydroxytryptophan were from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Naphthalene-2,3-dicarboxaldehyde (NDA) was from Invitrogen (Carlsbad, CA). L-$^{2}H_5$-Phenylalanine and $^{2}H_5$-indole-tryptophan were from Cambridge Isotope Co., MA. Dithiothreitol (DTT) was from Inalc (Milano, Italy). D,L-[4-^{2}H]-Phenylalanine and D,L-[3,5-$^{2}H_2$]-phenylalanine were synthesized as previously described (*12*). All other reagents were of the highest purity commercially available.

Construction of Vectors, Enzyme Expression and Purification. Site-directed mutagenesis of the catalytic domain of rat phenylalanine hydroxylase (Δ117PheH) was carried out with the Stratagene QuikChange Kit using *Pfu* DNA polymerase and the plasmid ptzRPH5 (*13*). Δ117PheH and the mutants E280A, F263A, and V379D were expressed and purified as previously described, with one modification (*13*, *14*). The ammonium sulfate pellet was resuspended in 30 mM potassium phosphate and 10% glycerol at pH 7.0 before loading onto the hydroxyapatite column. The enzymes were eluted from this column with a linear gradient of 30−450 mM potassium phosphate at pH 7.0, and stored in 80 mM Hepes/NaOH and 15% glycerol at pH 7.0. TrpH$_{102-416}$, a variant of rabbit tryptophan hydroxylase lacking 101 and 28 residues from the amino and carboxyl termini, respectively, was purified as previously described (*15*) with several modifications. The plasmid pEWOHΔ101ΔH was used to transform *E. coli* strain C41(DE3), a strain derived from BL21(DE3), which grows to a higher cell density (*16*). A single colony was used to inoculate 100 mL of LB broth (100 μg/mL of ampicillin) and allowed to grow at 37 °C for 5 h; 10 mL of the 100 mL culture was used to inoculate 1 L of LB broth (100 μg/mL of ampicillin). The culture was grown until the cell density reached an A_{600} of 0.9. The temperature was decreased to 18 °C, and the cells were permitted to grow until the cell density reached an A_{600} of 1.2. At this point, isopropyl-β-thiogalactoside was added to a final concentration of 100 μM. After 12 h, the cells were

harvested by centrifugation as previously described (*15*). The cells were resuspended in an 8-fold volume with respect to the cell weight of 80 mM Hepes/NaOH, 100 mM ammonium sulfate, and 2 mM DTT at pH 7.1. Nucleic acids were removed by addition of polyethyleneimine to a final concentration of 0.1%. Prior to the hydroxyapatite column, the ammonium sulfate pellet was resuspended in 50 mM potassium phosphate, 10% glycerol, 100 mM ammonium sulfate and 2 mM DTT at pH 7.1. The enzyme was then eluted with a linear gradient of 50−400 mM potassium phosphate, 10% glycerol, 100 mM ammonium sulfate, and 2 mM DTT at pH 7.1. The purified enzyme was stored in 15% glycerol, 100 mM ammonium sulfate, 2 mM DTT, and 50 mM Hepes/NaOH at pH 7.0, after the addition of a stoichiometric amount of ferrous ammonium sulfate.

Enzyme Assays. Tyrosine formation from phenylalanine was measured by monitoring the initial rate of the absorbance change at 275 nm due to the formation of tyrosine (ϵ_{275} = 1.34 mM^{-1} cm^{-1}) (*13*, *17*). The assays contained 0.2 mM 6-$MePH_4$ and 10−800 μM L-phenylalanine in 60 μg/mL catalase, 5 mM DTT, 15 μM ferrous ammonium sulfate, and 80 mM Hepes/NaOH at pH 7.0, at 25 °C. When racemic phenylalanine was used, the concentration range was 20−1600 μM. Control experiments with D-phenylalanine established that concentrations of the nonphysiological stereoisomer up to 1 mM had no effect on the activity of any of the enzymes.

Initial rates of 6-$MePH_4$ oxidation were determined using a coupled assay with DHPR and NADH, monitoring the decrease in absorbance at 340 nm due to the oxidation of NADH (ϵ_{275} = 6.22 mM^{-1} cm^{-1}) (*13*, *18*). The assay conditions were 10−800 μM L-phenylalanine for Δ117PheH and $TrpH_{102-416}$ and 10−2000 μM L-phenylalanine for Δ117PheH V379D, 0.05−1 μM enzyme, 100−200 μM 6-$MePH_4$, 60 μg/mL of catalase, 200−250 μM NADH, 0.2−0.5 units/mL DHPR, 15 μM ferrous ammonium sulfate, and 80 mM Hepes/NaOH at pH 7.0, at 25 °C.

Initial rates of formation of 5-hydroxytryptophan from tryptophan were monitored on a Applied Photophysics SX-18MV stopped-flow fluorometer (*19*). The sample was excited at 315 nm along a 10 mm path, and the emission was passed through a 335 nm cutoff filter. The assay conditions were 20−2000 μM L-tryptophan, 200 μM 6-$MePH_4$, 1.5 μM Δ117PheH, 7 mM DTT, 50 μg/mL of catalase, 10 μM ferrous ammonium sulfate, and 80 mM Hepes/NaOH at pH 7.0, at 25 °C. These conditions were obtained by mixing equal volumes from two syringes. Syringe 1 contained 400 μM 6-$MePH_4$, 12 mM DTT, tryptophan, and 80 mM Hepes/NaOH at pH 7.0. Syringe 2 contained 100 μg/mL of catalase, 3 μM Δ117PheH, 20 μM ferrous ammonium sulfate, 2 mM DTT, and 80 mM Hepes/NaOH at pH 7.0. The amount of 5-hydroxytryptophan formed was determined by comparison with a standard curve obtained under the same conditions.

To directly measure the stoichiometry of amino acid hydroxylation relative to that of 6-$MePH_4$ oxidation, an HPLC-based assay similar to that described previously (*20*, *21*) was performed. The conditions were 1.2 mM D,L-phenylalanine or 500 μM L-phenylalanine, 10 μM enzyme, 40−100 μM 6-$MePH_4$, and 25 mM potassium phosphate at pH 7.0, at 30 °C in 100 μL. The reactions were initiated with 6-$MePH_4$; after 3 min, they were quenched with 400 μL of 10 mM sodium borate at pH 9.2. To this were added 100 μL of 10 mM sodium cyanide and 40 μL of 10 mM naphthalene-2,3-dicarboxaldehyde (NDA) to form the 1-cyanobenz[*f*]-isoindole (CBI) derivatives (*22*). Then, 50 μL of the reaction were injected onto a Nova-Pak C18 column (2.1 × 150 mm). The CBI derivatives of tyrosine and phenylalanine were separated with a gradient of 30−35% acetonitrile and 70−65% 12.5 mM potassium phosphate and 1% THF at pH 6.8, with fluorescence detection using excitation and emission wavelengths of 420 and 490 nm, respectively. The amount of tyrosine was quantified by comparison with a standard curve obtained under the same conditions. Samples for mass spectrometry were made following the same protocol, except that 4 mM 6-$MePH_4$, 8 mM [4-^{2}H]- or [3,5-$^2H_2$]- phenylalanine, 100 μM enzyme, and 100 μM ferrous ammonium sulfate in 300 μL were used, and the reaction was quenched by the addition of 100 μL of 100 mM sodium borate at pH 9.2. Then, 50 μL of 100 mM sodium cyanide and 100 μL of 50 mM NDA were added, and the precipitated protein was removed by centrifugation. The entire reaction mixture was then injected onto the Nova-Pak C18 column, and the CBI derivatives of tyrosine and phenylalanine were separated with an isocratic mobile phase of 85% water and 15% acetonitrile. The peak corresponding to tyrosine eluted at 18 min and was detected with excitation and emission wavelengths of 470 and 590, nm respectively. The CBI-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. The ratios of the (*m*-1) peaks resulting from loss or retention of deuterium were corrected for ^{13}C contributions and used in calculations of the isotope effects.

Data Analysis. The Michaelis−Menten equation was used to determine k_{cat}, k_{cat}/K_m, and K_m values when initial rates were measured as a function of the concentration of a single substrate, using the program KaleidaGraph (Synergy Software, Reading, PA). Steady-state kinetic isotope effects were determined using Igor Pro (WaveMetrics, Lake Oswego, OR) to fit the data to eq 1. Equation 1 describes data for which there is an isotope effect only on the k_{cat} value. Here, v is the initial rate, k_{cat} is the turnover number, A is the substrate concentration, K_m is the Michaelis constant for the varied substrate, F_i is the mole fraction of deuterium in the substrate, and E_v is the isotope effect on k_{cat}. Isotope effects on the ratio of productive hydroxylation to total 6-$MePH_4$ consumption were calculated by direct comparison of the amount of tyrosine formed with phenylalanine versus deuterated phenylalanine.

$$v = \frac{k_{cat}A}{K_m + A^*(1 + F_i^*(E_v - 1))} \quad (1)$$

RESULTS

Steady-State Kinetics of Δ117PheH and Isotope Effects. The catalytic core of rat PheH, Δ117PheH, was chosen instead of the wild-type rat enzyme because the former does not need to be preactivated with phenylalanine, lysolecithin, or limited proteolysis, and it shows Michaelis−Menten kinetics (*13*). In addition, the stoichiometry of tyrosine formed with respect to 6-$MePH_4$ oxidized is one. The kinetic parameters for this enzyme reported here (Table 1) are not statistically different from values published previously. To

Table 1: Steady-State Kinetic Parameters for Δ117PheH and Δ117PheH V379D

	Δ117PheH		Δ117PheH V379D	
parameter	tyrosine formation[a]	6-MePH$_4$ oxidation[b]	tyrosine formation[a]	6-MePH$_4$ oxidation[b]
k_{cat} (min^{-1})	513 ± 23	471 ± 50	18 ± 1	67 ± 4
K_{phe} (μM)	120 ± 18	127 ± 53	420 ± 48	625 ± 85
k_{cat}/K_{phe} (μM^{-1} min^{-1})	4.3 ± 0.7		0.043 ± 0.004	
$K_{6-MePH4}$[c] (μM)	83 ± 9		63 ± 9	

[a] Determined from initial rates of tyrosine formation in 80 mM Hepes/NaOH, 5 mM DTT, 15 μM ferrous ammonium sulfate, 60 μg/mL of catalase and 200 μM 6-MePH$_4$ at pH 7.0, at 25 °C. [b] Determined from initial rates of 6-MePH$_4$ oxidation in 80 mM Hepes/NaOH, 15 μM ferrous ammonium sulfate, 60 μg/mL of catalase, 200−250 μM NADH, 0.2−0.5 units of DHPR and 200 μM 6-MePH$_4$ at pH 7.0, 25 °C. [c] Determined from initial rates of tyrosine formation in 80 mM Hepes/NaOH, 5 mM DTT, 15 μM ferrous ammonium sulfate, 60 μg/mL of catalase, and 500 μM L-phenylalanine for Δ117PheH or 1000 μM phenylalanine for Δ117PheH V379D at pH 7.0, at 25 °C.

determine if hydroxylation is rate-limiting in Δ117PheH as is the case in TrpH (*8*), isotope effects on tyrosine formation were measured using ring-deuterated phenylalanines; the results are summarized in Table 2. The data fit best to eq 1, in which there is an isotope effect on only k_{cat}. With Δ117PheH, there are significant normal kinetic isotope effects with [4-^{2}H]-, [3,5-$^2H_2$]-, and $^2H_5$-phenylalanine, in agreement with previous results for the wild-type rat enzyme (*11*). In the case of $^2H_5$-phenylalanine, the isotope effect is greater than the isotope effects obtained with [4-^{2}H]- and [3,5-$^2H_2$]-phenylalanine (Table 2).

Characterization of Mutant Enzymes and Kinetic Isotope Effects. In the case of TyrH, the active site mutants E326A and F309A, in which tetrahydropterin oxidation is largely uncoupled from amino acid hydroxylation, show inverse kinetic isotope effects on both steady-state kinetics and the stoichiometry, in contrast to the wild-type enzyme (*10*). The homologous mutations E280A and F263A were prepared in Δ117PheH, yielding enzymes that were 30% and 15% coupled with respect to tetrahydropterin oxidation. Kinetic isotope effects of unity were found on the ratio of amino acid hydroxylation to 6-MePH$_4$ consumption for all of the enzymes. These results suggest that the uncoupling occurred prior to the formation of the ferryl-oxo intermediate for these enzymes (vide infra). In the case of E280A, the kinetic isotope effects on initial rates of tyrosine formation with [4-^{2}H]-, [3,5-$^2H_2$]-, and $^2H_5$-phenylalanine as substrates were normal and comparable to those with Δ117PheH (results not shown).

The V379D mutation was initially identified in a screen for PheH active site mutants with an increased ability to hydroxylate tyrosine (*14*). Val379 does not interact directly with the amino acid substrate, but crystal structures of PheH with amino acids bound indicate that valine 379 is in the active site, in close proximity to the amino acid substrate (*25*, *26*). As is the case with the intact protein (*14*), the mutation of Val379 to aspartate affects several kinetic parameters for the hydroxylation of phenylalanine by Δ117PheH (Table 1). The k_{cat} value is 30-fold lower, the k_{cat}/K_{phe} value is about 100-fold lower, and there is a 3-fold increase in the K_{phe} value. The $K_{6-MePH4}$ value is not altered significantly by the mutation, suggesting that the mutation of valine 379 has little effect on the binding of the tetrahydropterin. For Δ117PheH V379D the k_{cat} value for 6-MePH$_4$ oxidation is about four times that for tyrosine formation, consistent with only 27% of the total 6-MePH$_4$ being consumed in productive hydroxylation. In contrast to Δ117PheH, Δ117PheH V379D shows a significantly inverse isotope effect with [4-^{2}H]-phenylalanine as substrate (Table 2). The value matches those obtained with mutant TyrH proteins and with wild-type TrpH (*8*, *10*). This suggests that the three aromatic amino acid hydroxylases share the same chemical mechanism for aromatic hydroxylation, but the isotope effect is masked in PheH by other chemical steps. An inverse isotope effect was only obtained when the site of hydroxylation was deuterated, suggesting that a change in hybridization from sp^2 to sp^3 occurs exclusively at the para position. When [3,5-$^2H_2$]-phenylalanine is used as a substrate for Δ117PheH V379D, there is a normal deuterium isotope effect; the value is identical within experimental error to that for 117PheH.

Isotope Effects on the Stoichiometry of Amino Acid Hydroxylation and 6-MePH$_4$ Oxidation. The stoichiometry of the 6-MePH$_4$ oxidized relative to that of the tyrosine formed was determined by measuring the amount of tyrosine after a limiting amount of 6-MePH$_4$ had been consumed. The changes in stoichiometry when [4-^{2}H]-, [3,5$^2H_2$]-, and $^2H_5$-phenylalanines are used as substrates for Δ117PheH and Δ117PheH V379D yield the isotope effects on the stoichiometry in Table 3. There was no isotope effect on the

Table 2: Kinetic Isotope Effects on the Hydroxylation of Deuterated Phenylalanines[a]

enzyme	4-^{2}H-phenylalanine	3,5-$^2H_2$-phenylalanine	$^2H_5$-phenylalanine
Δ117PheH	1.19 ± 0.06	1.12 ± 0.04	1.41 ± 0.05
Δ117PheH V379D	0.92 ± 0.03	1.07 ± 0.05	0.98 ± 0.09
TrpH$_{102-416}$	0.96 ± 0.07	1.04 ± 0.06	1.01 ± 0.04

[a] Conditions are the same as those for Table 1.

Table 3: Kinetic Isotope Effects on the Stoichiometry of Tyrosine Formation[a]

enzyme	4-^{2}H-phenylalanine	3,5-$^2H_2$-phenylalanine	$^2H_5$-phenylalanine
Δ117PheH	1.01 ± 0.03	1.00 ± 0.07	1.01 ± 0.02
Δ117PheH V379D	0.96 ± 0.03	1.06 ± 0.05	0.93 ± 0.04

[a] Assay conditions: 25 mM potassium phosphate at pH 7.0, 500 μM L-phenylalanine, or 1.2 mM D,L-phenylalanine, 10 μM enzyme, and 40−100 μM 6-MePH$_4$ at 30 °C.

Table 4: Ratio of Deuterium to Hydrogen in Tyrosine upon Hydroxylation of Deuterated Phenylalanine[a]

enzyme	4-^{2}H-phenylalanine	3,5-$^2H_2$-phenylalanine
Δ117PheH	3.79 ± 0.19	6.81 ± 0.46
Δ117PheH V379D	3.68 ± 0.12	6.27 ± 0.26

[a] Conditions: 25 mM potassium phosphate at pH 7.0, 100 μM ferrous ammonium sulfate, 8 mM D,L-phenylalanine, 100 μM enzyme, and 4 mM 6-MePH$_4$ at 30 °C. Amino acid products were purified by HPLC as the N-substituted cyanobenz[*f*]isoindole derivatives. Deuterium content was determined by negative mode ESI mass spectrometry.

stoichiometry with Δ117PheH, consistent with the ratio of 6-MePH$_4$ oxidation to amino acid hydroxylation being one. In contrast, inverse isotope effects on the stoichiometry were found for Δ117PheH V379D with both [4-^{2}H]- and $^2H_5$-phenylalanine as substrates. Both isotope effects match the isotope effect on k_{cat} obtained with [4-^{2}H]-phenylalanine.

Deuterium Content of Products. The deuterium contents of the tyrosine products arising from [4-^{2}H]- and [3,5$^2H_2$]-phenylalanine were determined by mass spectrometry. When [4-^{2}H]- phenylalanine is used as substrate for Δ117PheH (Table 4), about 75% of the deuterium in the substrate is retained in the product, in excellent agreement with previous results for the wild-type nonrecombinant enzyme (*27*, *28*). In the case of [3,5-$^2H_2$]-phenylalanine, about one-eighth of the product has lost one of the two deuterium atoms present in the substrate. The mutation of Val379 to aspartate has no effect on the deuterium retention in the products (Table 4), suggesting that steps after oxygen addition to the phenyl ring of phenylalanine are not significantly altered in the mutant protein.

Tryptophan as a Substrate for Δ117PheH. Tryptophan is a good substrate for wild-type PheH (*29*, *30*). Δ117PheH has a k_{cat} value of 33 ± 2 min^{-1} and a K_{trp} value of 700 ± 80 μM when 5-hydroxytryptophan formation is followed and values of 47 ± 6 min^{-1} and 1000 ± 290 μM for 6-MePH$_4$ oxidation. This yields a stoichiometry of 0.70 ± 0.10, suggesting that 6-MePH$_4$ oxidation and tryptophan hydroxylation are mostly coupled. The use of $^2H_5$-indole-tryptophan as a substrate for Δ117PheH yields a $^Dk_{cat}$ value of 0.89 ± 0.04, consistent with a change of hybridization from sp^2 to sp^3 at the transition state for an electrophilic aromatic substitution reaction (*8*). The inverse isotope effect on the k_{cat} value is consistent with the rate constant for the reaction of the hydroxylating intermediate and the indole ring of tryptophan being slower than all other first-order steps in Δ117PheH.

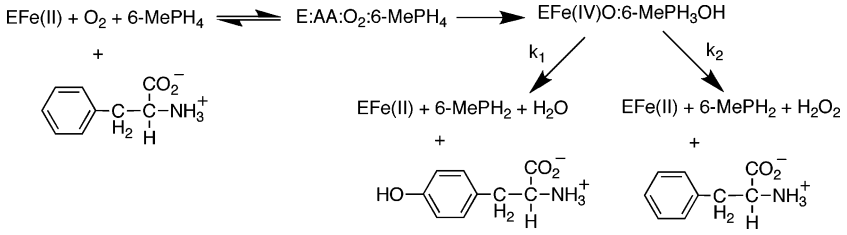
Kinetic Isotope Effects with Phenylalanine as a Substrate for TrpH$_{102-416}$. Phenylalanine is also a substrate for TrpH$_{102-416}$ (*15*). In the present work, k_{cat} values of 127 ± 6 min^{-1} for tyrosine formation and 131 ± 14 min^{-1} for 6-MePH$_4$ oxidation were found; these are somewhat higher than previously published results (*15*). Ring-deuterated phenylalanines were also used as substrates for TrpH$_{102-416}$; the resulting isotope effects are given in Table 2. TrpH$_{102-416}$ shows considerable substrate inhibition at phenylalanine concentrations higher than 200 μM, greatly limiting the precision of the $^Dk_{cat}$ value. Despite the lack of precision, the values for TrpH$_{102-416}$ are clearly in better agreement with those for Δ117PheH V379D.

DISCUSSION

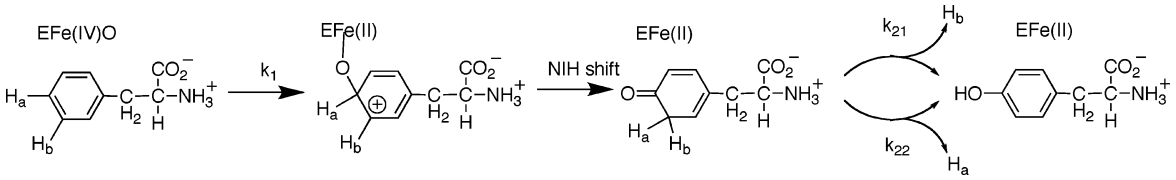
The results presented here provide evidence that all three aromatic amino acid hydroxylases share the same chemical mechanism but differ in the identity of the rate-limiting step. In the case of TrpH, the inverse deuterium kinetic isotope effect on k_{cat} and the sensitivity of k_{cat} to the amino acid substrate establish that hydroxylation of the indole ring of tryptophan is rate-limiting (*8*, *31*). In the case of wild-type TyrH, the lack of an isotope effect on k_{cat} with [3,5-$^2H_2$]-tyrosine, ^{18}O kinetic isotope effects, and the insensitivity of k_{cat} to the amino acid substrate suggest that oxygen activation is completely rate-limiting (*9*, *32*, *33*). In the case of PheH, a kinetic isotope effect of 1.45 with $^2H_5$-phenylalanine previously suggested a different rate-limiting step and possibly a different chemical mechanism for PheH (*11*).

In the present study, it was possible to unmask the isotope effect on hydroxylation in the PheH reaction using a mutant enzyme, Δ117PheH V379D. The theory of unmasking kinetic isotope effects by the partitioning of intermediates via branched pathways has been described previously (*10*, *34*, *35*). Scheme 3 illustrates the basic kinetic scheme for the analysis. The enzyme binds all three substrates and proceeds through the first irreversible step to form a high-valence ferryl-oxo intermediate, EFe(IV)O. With some amino acid substrates or mutant proteins, there is a branch point after the formation of this hydroxylating intermediate, which leads to unproductive consumption of tetrahydropterin. In such a case, the reaction can follow either the productive hydroxylation pathway with net rate constant k_1 or the unproductive pathway with net rate constant k_2. If it is assumed that hydroxylation and unproductive breakdown of the intermediate are irreversible events, which is a reasonable assumption, rate constant k_1 will be sensitive to deuterium substitution on the amino acid, and k_2 will not. The stoichiometry of tyrosine formed relative to 6-MePH$_4$ consumed is then $k_1/(k_1 + k_2)$. Deuterium substitution on the amino acid can change the net rate constant k_1, thus changing the stoichiometry. If $k_2 \gg k_1$ the isotope effect on the stoichiometry will yield the intrinsic isotope effect directly; the isotope effect will also be expressed on the k_{cat} value because the change in the contribution of the unproductive pathway will result in a change in the rate of formation of the hydroxylated amino acid (*10*, *35*).

Scheme 3



Scheme 4



Mutating Val379 in Δ117PheH alters the stoichiometry of the reaction so that more 6-MePH$_4$ is oxidized relative to the amount of tyrosine formed. This is consistent with the introduction of a branched pathway in which the high-valence ferryl-oxo intermediate is breaking down unproductively more frequently than it reacts with the aromatic ring. This mutant enzyme also exhibits an inverse isotope effect, consistent with the predictions of Schemes 2 and 3. An inverse isotope effect is seen only when deuterium is present at the site of hydroxylation, establishing that attack of the hydroxylating intermediate occurs exclusively at the para position of the amino acid and ruling out the formation of an arene intermediate as was once proposed for PheH (*36*). The inverse isotope effect with Δ117PheH V379D agrees well with those previously reported for TyrH and TrpH, establishing that the three aromatic amino acid hydroxylases share similar transition states for aromatic hydroxylation. Thus, the isotope effect for the reaction of the hydroxylating intermediate with the phenyl ring of phenylalanine in the wild-type enzyme is masked by another isotope-sensitive step.

The minimal mechanism of Scheme 3 predicts that the isotope effect on k_{cat} will vary between one and the intrinsic inverse isotope effect if the isotope effect is due solely to the initial reaction of the hydroxylating intermediate with the amino acid substrate to form the new carbon−oxygen bond. However, for the wild-type enzyme and Δ117PheH, normal isotope effects are seen with ring-deuterated phenylalanines. A normal isotope effect will occur in a step in which a C−D bond is cleaved; for the mechanism of Scheme 2, there are two such steps: the NIH shift and the subsequent tautomerization to form the aromatic amino acid product. Computational studies done on PheH are consistent with the mechanism of Scheme 2, with the initial cation undergoing a zero-barrier NIH shift to form a dienone intermediate (*37*). This will result in the NIH shift being fast and isotope-insensitive. The same computational studies indicate that the subsequent tyrosine formation from the dienone is associated with a large energy barrier so that this step will be isotope-sensitive; this conclusion is confirmed by the retention of deuterium in the product tyrosine shown in Table 4. The normal $^{D}k_{cat}$ values with Δ117PheH can then be attributed to the isotope-sensitive tautomerization of the amino acid being partially rate limiting for the overall reaction.

The results presented here can be used to estimate the relative values of the rate constants for hydroxylation and the subsequent tautomerization. Scheme 4 shows a minimal mechanism for the reaction of a hydroxylating intermediate with the aromatic ring of phenylalanine to form tyrosine. In Scheme 4, the ferryl-oxo intermediate reacts with the amino acid substrate with rate constant k_1 to form a cationic intermediate. The subsequent NIH shift is likely to be fast and isotope-insensitive, as noted above. The rate constants k_{21} and k_{22} are for the possible cleavages of the two different CH bonds during tautomerization and are subject to primary kinetic isotope effects. Equations 2 and 3 relate the isotope effects on k_{22} and k_{21} to $^{D}k_{cat}$ for [4-^{2}H]- and [3,5-^{2}H$_2$]-phenylalanine, respectively.[2]

$$^{D}k_{cat} = \frac{\dfrac{^{D}k_{22}\left(1+\dfrac{k_{21}}{k_{22}}\right)}{1+{}^{D}k_{22}\left(\dfrac{k_{21}}{k_{22}}\right)} + \dfrac{^{D}k_{1}}{k_{1}}(k_{22}+k_{21})}{1+\dfrac{^{D}k_{1}}{k_{1}}(k_{22}+k_{21})} \quad (2)$$

$$^{D}k_{cat} = \frac{\dfrac{^{D}k_{21}\left(1+\dfrac{k_{22}}{k_{21}}\right)}{1+{}^{D}k_{21}\left(\dfrac{k_{22}}{k_{21}}\right)} + \dfrac{k_{21}+k_{22}}{k_{1}}}{1+\dfrac{k_{22}+k_{21}}{k_{1}}} \quad (3)$$

The magnitudes of $^{D}k_{21}$, $^{D}k_{22}$, and k_{21}/k_{22} can be estimated from the isotopic contents of the tyrosine produced from [4-^{2}H]- and [3,5-^{2}H$_2$]-phenylalanine. The ratios of tyrosine formed by loss of hydrogen versus deuterium are related to the rate constants and the isotope effects by eqs 4 and 5. In the case of [4-^{2}H]-phenylalanine, H$_a$ is deuterium so that loss of deuterium with rate constant k_{22} will exhibit an isotope effect. Accordingly, the ratio R_1 of the product containing one deuterium (P_D) to that containing only hydrogen (P_H) is given by eq 4. Similarly, for [3,5-^{2}H$_2$]-phenylalanine, where H$_b$ is deuterium, the ratio R_2 of the product with two deuteriums to that with one is given by eq 5. Values of R_1 and R_2 were determined by mass spectrometry and are given in Table 4.

$$P_D/P_H = R_1 = {}^{D}k_{22}(k_{21}/k_{22}) \quad (4)$$

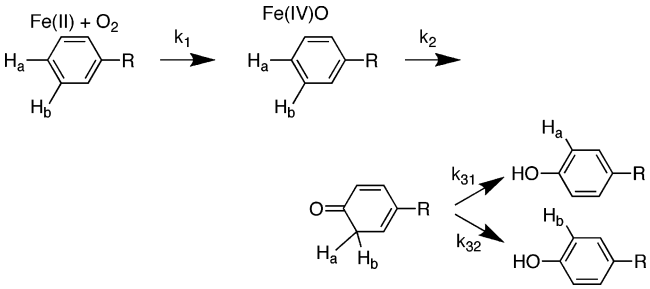
$$P_{D2}/P_D = R_2 = {}^{D}k_{21}(k_{22}/k_{21}) \quad (5)$$

These two equations contain three unknowns, $^{D}k_{21}$, $^{D}k_{22}$, and k_{21}/k_{22} and cannot be solved explicitly with the data given here. However, they can be solved in two limiting cases. If k_{21} equals k_{22}, then $^{D}k_{22}$ equals R_1, and $^{D}k_{21}$ equals R_2. In this case, if $^{D}k_1$, the intrinsic isotope effect on k_1, is taken as 0.92 ± 0.03, then the $^{D}k_{cat}$ value of 1.19 ± 0.06 for [4-^{2}H]-phenylalanine and eq 2 yield a value for k_{21}/k_1 of 2.6 ± 1.1. A similar analysis can be done for [3,5-^{2}H$_2$]-phenylalanine using eq 3, to obtain a k_{21}/k_1 value of 1.1 ± 0.6. Equations 4 and 5 can also be used to obtain the isotope effects and ratios of rate constants if both k_{21} and k_{22} are affected to the

[2] The derivations of eqs 2 and 3 are given in the Supporting Information.

same extent by deuterium substitution, that is, $^{D}k_{21}$ equals $^{D}k_{22}$. Then, the isotope effect on either is given by $\sqrt{R_1R_2}$; the data of Table 4 for Δ117PheH give a value for this isotope effect of 5.1 ± 0.3. In addition, if $^{D}k_{21}$ equals $^{D}k_{22}$, then k_{21}/k_{22} equals $\sqrt{R_1/R_2}$, 0.75 ± 0.04. Under these conditions, eq 2 and the $^{D}k_{cat}$ value for [4-^{2}H]-phenylalanine yield a value for k_{21}/k_1 of 1.6 ± 0.9, whereas eq 3 and the $^{D}k_{cat}$ value for [3,5-^{2}H$_2$]-phenylalanine yield a value for k_{21}/k_1 of 1.4 ± 1.0. The values for the second case are in better agreement, suggesting that $^{D}k_{21}$ and $^{D}k_{22}$ are equivalent, but there is a slight (~34%) preference for the loss of the hydrogen originally present at C4. Both analyses are consistent with the overall rate constant for tautomerization ($k_{21} + k_{22}$) being about 4-fold (3.8 ± 2.2) faster than that for the initial reaction of the hydroxylating intermediate with the aromatic ring.[3]

[3] This treatment explicitly assumes that the formation of the hydroxylating intermediate is rapid. Scheme 5 is a more complete description of the reaction, which includes formation of the hydroxylating intermediate with rate constant k_1 followed by oxygen addition with rate constant k_2.



This is equivalent to Scheme 4 with k_1 of eqs 2 and 3 encompassing the first two steps. Equations 7 and 8 can, thus, be derived by substitution of $k_1k_2/(k + k_2)$ for k_1 and of k_{31} and k_{32} for k_{21} and k_{22}.

$$^{D}k_{cat} = \frac{\dfrac{^{D}k_{31}\left(1+\dfrac{k_{32}}{k_{31}}\right)}{1+{}^{D}k_{31}\left(\dfrac{k_{32}}{k_{31}}\right)} + \dfrac{k_{31}+k_{32}}{\dfrac{k_1k_2}{k_1+k_2}}}{1+\dfrac{k_{31}+k_{32}}{\dfrac{k_1k_2}{k_1+k_2}}} \quad (7)$$

$$^{D}k_{cat} = \frac{\dfrac{^{D}k_{32}\left(1+\dfrac{k_{31}}{k_{32}}\right)}{1+{}^{D}k_{32}\left(\dfrac{k_{31}}{k_{32}}\right)} + \dfrac{^{D}k_2(k_{31}+k_{32})(k_1+k_2)}{k_1k_2}}{1+\dfrac{^{D}k_2(k_{31}+k_{32})(k_1+k_2)}{k_1k_2}} \quad (8)$$

Neither eq 7 nor 8 can be explicitly solved with the available data. Thus, one cannot explicitly consider the rate constant for oxygen activation in the calculations of ratios of rate constants. Instead, the analyses in the text yield the ratio of k_{31} or k_{32} to the net rate constant for oxygen activation plus attack of the hydroxylating intermediate. Equations 7 and 8 simplify to eqs 2 and 3 under two conditions. First, if oxygen activation is much slower than the subsequent attack, k_1 in eqs 2 and 3 is the rate constant for oxygen activation and $^{D}k_1 = 1$. This has only a small effect on the calculation for 4-^{2}H$_2$-phenylalanine, yielding a value for k_{21}/k_1 of 1.5 ± 0.8, and no effect on the calculation for 3,5-^{2}H$_2$-phenylalanine. Second, if oxygen activation is much faster, the rate constant for oxygen activation drops out of eqs 7 and 8, so that eqs 2 and 3 apply explicitly.

The relative values of the tautomerization and hydroxylation steps can also be calculated from the $^{D}k_{cat}$ value for [^{2}H$_5$]-phenylalanine by using eq 6 and 5.1 and 0.92 as the intrinsic isotope effects for these steps. This yields a value for $(k_{21} + k_{22})/k_1$ of 7.5 ± 1.0. Thus, the $^{D}k_{cat}$ values for all three ring-deuterated phenylalanines are consistent with tautomerization being 6−7-fold faster than hydroxylation.

$$^{D}k_{cat} = \frac{^{D}(k_{22}+k_{21}) + {}^{D}k_1\left(\dfrac{k_{22}+k_{21}}{k_1}\right)}{1+\dfrac{k_{22}+k_{21}}{k_1}} \quad (6)$$

In order to determine if the normal $^{D}k_{cat}$ values with phenylalanine as a substrate for Δ117PheH are solely dependent on the substrate, the enzyme, or both, isotope effects were determined with TrpH$_{102-416}$ using phenylalanine as a substrate and with Δ117PheH using tryptophan as a substrate. With ^{2}H$_5$ -tryptophan as a substrate for Δ117PheH, there is an inverse isotope effect on $^{D}k_{cat}$ equal to that seen with TrpH (*8*). The stoichiometry shows that 6-Me-PH$_4$ oxidation is well coupled with amino acid hydroxylation; thus, $k_1 > k_2$ in Scheme 3. This suggests that tautomerization of a common intermediate is no longer rate limiting when tryptophan is the substrate for Δ117PheH. The 18-fold lower k_{cat} value when tryptophan is the substrate and the inverse isotope effect on $^{D}k_{cat}$ establish that the reaction of the ferryl-oxo intermediate with the indole ring of tryptophan is rate limiting in Δ117PheH, as is the case for TrpH (*15*). The identity of the rate-limiting step is clearly substrate dependent. If the rate constants for hydroxylating intermediate attack were dependent exclusively on the intrinsic reactivity of the amino acid, one would expect an inverse $^{D}k_{cat}$ value with phenylalanine and not tryptophan because the latter would be more reactive in an electrophilic aromatic substitution. The simplest explanation is that interactions of the enzyme with bound tryptophan are different from those with phenylalanine.

While the substrate inhibition with phenylalanine as a substrate for TrpH$_{102-416}$ greatly limited the precision with which the isotope effects could be measured, the $^{D}k_{cat}$ values are clearly smaller with deuterated phenylalanine as substrates for TrpH$_{102-416}$ than is the case with Δ117PheH. This suggests that the reaction of the ferryl-oxo intermediate with the amino acid substrate is slower in TrpH$_{102-416}$ than Δ117PheH and may be rate limiting with phenylalanine in addition to tryptophan.

The results presented here are consistent with the mechanism for aromatic amino acid hydroxylation shown in Scheme 2 for PheH. Tetrahydropterin, molecular oxygen, and amino acid bind to the active site of the enzyme, triggering the activation of molecular oxygen to form a high valence ferryl-oxo intermediate. Next, this intermediate reacts with the amino acid, forming a cationic intermediate. An NIH shift occurs, resulting in high retention of the deuterium initially present at the site of hydroxylation. Last, a partially rate-limiting proton loss from a tetrahedral intermediate occurs to regenerate the ferrous form of the enzyme and the hydroxylated product. The results presented here show that the transition state structure for aromatic hydroxylation is similar for the three enzymes and provide estimates of the

relative values of individual rate constants in the reaction of PheH.

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SUPPORTING INFORMATION AVAILABLE

Derivations of eqs 2 and 3. This material is available free of charge via the Internet at http://pubs.acs.org

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