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Mutagenesis of a Specificity-Determining Residue in Tyrosine Hydroxylase Establishes That the Enzyme Is a Robust Phenylalanine Hydroxylase but a Fragile Tyrosine Hydroxylase

S. Colette Daubner,*[,†] Audrey Avila,[†] Johnathan O. Bailey,[‡] Dimitrios Barrera,[§] Jaclyn Y. Bermudez,[†] David H. Giles,[∥] Crystal A. Khan,[∥] Noel Shaheen,[†] Janie Womac Thompson,[‡] Jessica Vasquez,[‡] Susan P. Oxley,[§] and Paul F. Fitzpatrick*[,∥]

[†]Department of Biological Sciences, St. Mary's University, San Antonio, Texas 78228, United States

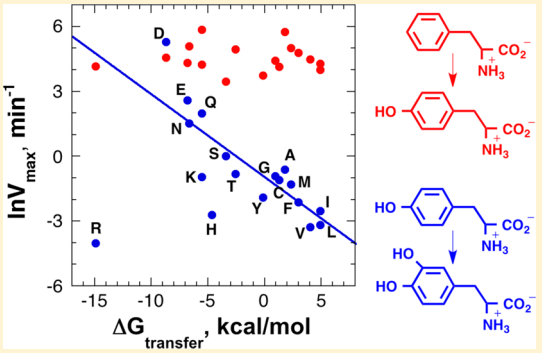
[‡]Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77840, United States

[§]Department of Chemistry and Biochemistry, St. Mary's University, San Antonio, Texas 78228, United States

[∥]Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229, United States

Supporting Information

ABSTRACT: The aromatic amino acid hydroxylases tyrosine hydroxylase (TyrH) and phenylalanine hydroxylase (PheH) have essentially identical active sites; however, PheH is nearly incapable of hydroxylating tyrosine, while TyrH can readily hydroxylate both tyrosine and phenylalanine. Previous studies have indicated that Asp425 of TyrH is important in determining the substrate specificity of that enzyme [Daubner, S. C., Melendez, J., and Fitzpatrick, P. F. (2000) *Biochemistry* 39, 9652−9661]. Alanine-scanning mutagenesis of amino acids 423−427, a mobile loop containing Asp425, shows that only mutagenesis of Asp425 alters the activity of the enzyme significantly. Saturation mutagenesis of Asp425 results in large (up to 10^4) decreases in the V_{max} and V_{max}/K_{tyr} values for tyrosine hydroxylation, but only small decreases or even increases in the V_{max} and V_{max}/K_{phe} values for phenylalanine hydroxylation. The decrease in the tyrosine hydroxylation activity of the mutant proteins is due to an uncoupling of tetrahydropterin oxidation from amino acid hydroxylation with tyrosine as the amino acid substrate. In contrast, with the exception of the D425W mutant, the extent of coupling of tetrahydropterin oxidation and amino acid hydroxylation is unaffected or increases with phenylalanine as the amino acid substrate. The decrease in the V_{max} value with tyrosine as the substrate shows a negative correlation with the hydrophobicity of the amino acid residue at position 425. The results are consistent with a critical role of Asp425 being to prevent a hydrophobic interaction that results in a restricted active site in which hydroxylation of tyrosine does not occur.



The aromatic amino acid hydroxylases tyrosine hydroxylase (TyrH), phenylalanine hydroxylase (PheH), and tryptophan hydroxylase (TrpH) make up a small family of enzymes with important roles in metabolism. They are all rate-limiting enzymes for particular pathways: PheH for phenylalanine catabolism, TyrH for catecholamine synthesis, and TrpH for serotonin synthesis.[1] The reactions they catalyze are shown in Scheme 1. These enzymes all form tetramers of subunits that each have two domains, with the larger (∼340 residues) carboxyl-terminal catalytic domains showing considerable similarity in sequence and three-dimensional structure.[2,3] The smaller amino-terminal regulatory domains are of different lengths and show low levels of identity among the three enzymes. Gene analysis suggests that all three enzymes evolved from an ancient hydroxylase. TyrH branched off first, 750 million years ago, and TrpH and PheH diverged 600 million years ago; the enzymes acquired different amino termini some time after TyrH arose.[4,5]

The common mechanism proposed for the aromatic amino acid hydroxylases is shown in Scheme 2.[6] After the tetrahydropterin and the amino acid substrate bind, oxygen reacts to form a bridged peroxypterin; heterolytic cleavage of the proposed peroxy moiety would form the Fe(IV)═O hydroxylating intermediate and a 4a-hydroxypterin, the pterin product that is released by the enzyme. The Fe(IV)═O intermediate transfers an oxygen atom to the aromatic ring of the amino acid via electrophilic aromatic substitution.[7,8]

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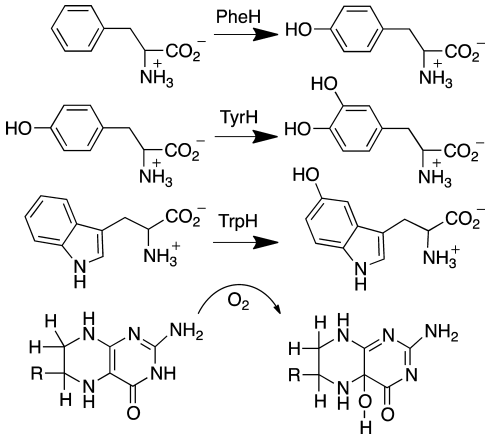
Scheme 1



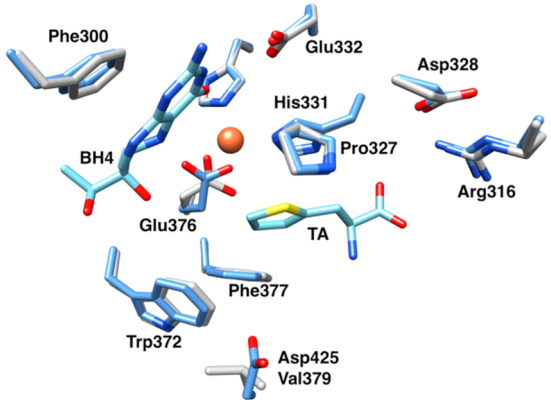


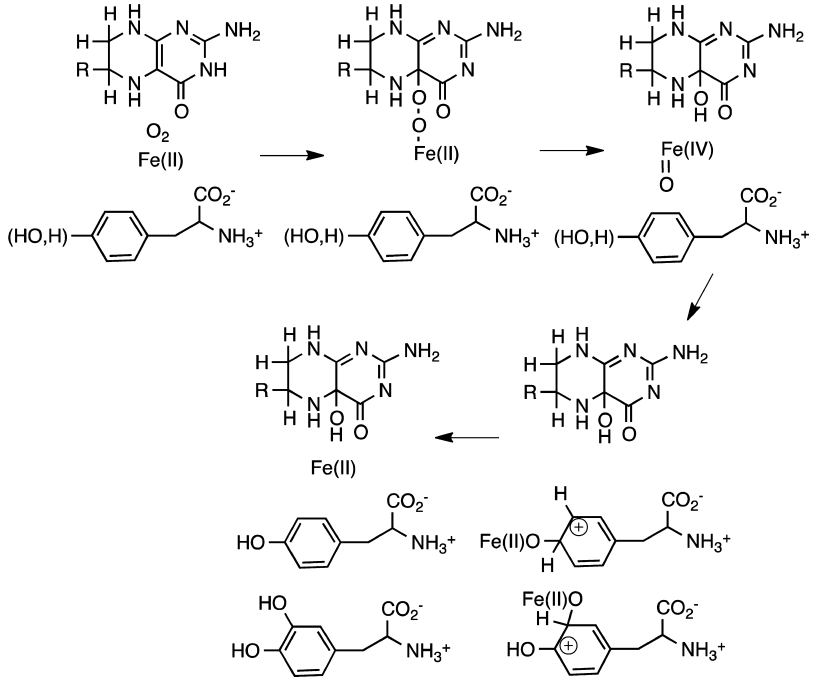
Figure 1. Comparison of the active sites of TyrH (blue carbons) and PheH (gray carbons). The residue numbers are for TyrH. Abbreviations: TA, thienylalanine; BH4, tetrahydrobiopterin. This figure was drawn using PDB entries 1TOH, 1KW0, and 1DMW and Chimera.[60]

The active sites of the aromatic amino acid hydroxylases are highly conserved.[9,10] Figure 1 shows an overlay of the active sites of TyrH and PheH to illustrate the similarity. The active sites of all three enzymes contain two histidines and a glutamate that bind the single active site iron (His331, His336, and Glu376, respectively, in TyrH),[11] a phenylalanine (Phe300 in TyrH)[12,13] and a glutamate (Glu332 in TyrH)[13,14] that bind the pterin, an arginine and the associated aspartate (Arg316 and Asp328, respectively, in TyrH)[14] that bind the carboxylate of the amino acid substrate, and several residues (Pro327, Phe377, and Trp372 in TyrH)[15] that form a hydrophobic chamber for the side chain of the amino acid substrate.

The three enzymes differ in the extent of their specificity for the amino acid substrate. TyrH can hydroxylate all three aromatic amino acids, with the following preference based on V_{max}/K_m values: tyrosine > phenylalanine > tryptophan (~5:1:0.2).[15,16] PheH is quite specific for phenylalanine; it can also hydroxylate tryptophan, but with a 6000-fold lower V_{max}/K_{trp} value,[16,17] and the extent of formation of 3,4-dihydroxyphenylalanine (DOPA) from tyrosine by PheH is so low that it is difficult to measure.[16,18] TrpH can hydroxylate both tryptophan and phenylalanine, with a preference for the physiological substrate of ~5-fold, but does not hydroxylate tyrosine.[15,19] The substrate specificities are determined by residues in the catalytic domains, in that mutant proteins lacking the regulatory domains and chimeric proteins that contain the regulatory domain of one hydroxylase attached to the catalytic domain of the other display the same substrate specificities as the native enzymes.[3]

The structural basis for the amino acid substrate specificities of these enzymes has been studied by site-directed mutagenesis of residues in the active site cleft that differ among the three enzymes. In general, the effects on substrate specificity have been modest, with changes in relative V_{max}/K_m values of an order of magnitude or less.[15,16,20,21] The exceptions have been

Scheme 2



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residues near the active site that do not interact directly with the substrates. The most dramatic is at position 425 in TyrH, which is homologous to position 379 of PheH; this is a conserved aspartate in TyrH, a conserved valine in PheH, and a conserved isoleucine in TrpH. The $(V_{max}/K_{phe})/(V_{max}/K_{tyr})$ value for TyrH D425V is ~50000-fold higher than that of wild-type TyrH.[16,18] Indeed, TyrH D425V is better than PheH at catalyzing the formation of tyrosine from phenylalanine based on the V_{max}/K_{phe} values but has V_{max} and V_{max}/K_{tyr} values for catalyzing the formation of DOPA from tyrosine that are less than 1% of the values for wild-type TyrH. Combining the reverse mutation in PheH, V379D, with a second mutation, H264Q, results in a PheH with significant activity at tyrosine hydroxylation and a 3000-fold decrease in its preference for phenylalanine over tyrosine as the substrate. Asp425 lies at the bend of a loop that reaches over the opening to the active site cleft in the structures of TyrH and PheH (Figure 2).

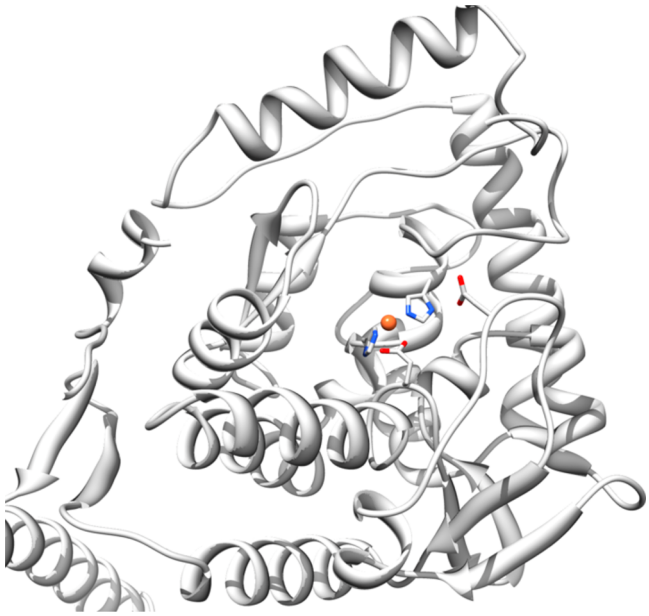

Figure 2. Ribbon drawing of TyrH (PDB entry 1TOH) with the iron ligands and Asp425 shown.

Examination of the dynamics of TyrH using hydrogen–deuterium exchange has shown that this loop is quite mobile.[22] In the work described here, we have conducted alanine scanning of residues in the loop and saturation mutagenesis of Asp425 to understand the role of this residue in TyrH.

MATERIALS AND METHODS

Materials. Custom oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases were from New England Biolabs Inc. (Beverly, MA). *Pfu* DNA polymerase was obtained from Stratagene USA (La Jolla, CA). DNA sequencing was performed using the ABI BigDye V3.0 kit of Life Technologies (Grand Island, NY). Plasmids were purified using kits from Qiagen Inc. (Valencia, CA). 6-Methyltetrahydropterin (6-MePH$_4$) was purchased from B. Schircks Laboratories (Jona, Switzerland). Leupeptin and pepstatin were obtained from Peptides International (Louisville, KY). *Escherichia coli* strain BL21(DE3) from EMD Millipore (Billerica, MA) was used for expression of TyrH, and Invitrogen's OmniMax strain from Life Technologies was used for DNA preparations and cloning. L-Tyrosine, DOPA, L-phenylalanine, glycerol, catalase, and dihydropteridine reductase were from Sigma-Aldrich Chemical Corp. (St. Louis, MO). Heparin-Sepharose CL-6B and phenyl-Sepharose Fast Flow were purchased from GE Healthcare (Piscataway, NJ). Ampicillin and chloramphenicol were from Affymetrix (Santa Clara, CA). HEPES and isopropyl β-thiogalactopyranoside (IPTG) were purchased from Research Products International Corp. (Mount Prospect, IL). Dithiothreitol was from Inalco (San Luis Obispo, CA).

Expression and Purification of Recombinant Proteins. The plasmid for expression of wild-type rat TyrH (pETYH8) has been described previously.[3,23] Mutagenesis was performed using the QuikChange site-directed mutagenesis method (Stratagene). For each construct, the entire coding region was sequenced to ensure that no other mutations were present. *E. coli* strain BL21(DE3) pLysS was used for expression in all cases. Expression of TyrH D425Q, -E, and -N was conducted in a manner similar to that used for the wild-type enzyme, with growth at 37 °C for 3 h after induction with 0.25 mM IPTG; all the other Asp425 variants were expressed by growing *E. coli* at 18 °C for 15 h after induction with 0.25 mM IPTG. In all cases, enzyme purification was conducted as described for the wild-type enzyme,[16] except that 1% streptomycin sulfate was used to precipitate nucleic acids. Concentrations of wild-type TyrH and variants were determined using an $A_{280}^{1\%}$ value of 1.04 and a mass of 56000 Da. Enzyme purity was assessed by denaturing polyacrylamide gel electrophoresis.[24]

Enzyme Assays. The formation of DOPA from tyrosine was measured using a colorimetric assay.[25] Standard conditions were 0.1 μM TyrH, 100 μM tyrosine, 400 μM 6-MePH$_4$, 100 μg/mL catalase, 10 μM ferrous ammonium sulfate, 1 μM dithiothreitol, 50 mM HEPES (pH 7.0), and 30 °C. Assays were conducted for 2 min and then reactions were quenched with HCl. For K_{tyr} determinations, the concentration of tyrosine was 5–4000 μM, depending on the variant.

For variants with a greatly weakened ability to hydroxylate tyrosine, high-performance liquid chromatography (HPLC) was used to determine the V_{max} for DOPA production. The tyrosine hydroxylation reaction was performed as described above, but for 5 min and with 5–10 μM enzyme, in quadruplicate, at a tyrosine concentration ≥8 times the K_{tyr} value determined using the tetrahydropterin oxidation assay (see below). (For TyrH D425K, because of the limited solubility of tyrosine, the concentration of tyrosine was set equal to the K_{tyr}, 2.1 mM, and the number of moles of DOPA formed was multiplied by 2.) The quenched reaction mixtures were centrifuged for 1 min, and 10 μL of the supernatants was applied to a Waters Atlantis C18 5 μm (4.6 mm × 250 mm) column. The column was eluted with a mobile phase of 30% methanol and 70% aqueous 0.5% trifluoroacetic acid at a flow rate of 0.7 mL/min; tyrosine eluted after DOPA. Tyrosine and DOPA were detected by fluorescence using a Waters 2475 Multi λ fluorescence detector. The amount of DOPA produced was determined by comparison with a standard curve.

The formation of tyrosine from phenylalanine was measured using a continuous assay, monitoring the absorbance change at 275 nm due to the production of tyrosine.[3] Standard assays contained 0.1 μM enzyme, 5–3000 μM phenylalanine, 200 μM 6-MePH$_4$, 100 μg/mL catalase, 1 μM dithiothreitol, 10 μM ferrous ammonium sulfate, and 80 mM Hepes (pH 7.0) at 30 °C.

6-MePH$_4$ oxidation rates were determined using a coupled assay with dihydropteridine reductase, monitoring the decrease in absorbance at 340 nm due to NADH oxidation.[26] The assays contained 5−2000 μM phenylalanine or 5−1300 μM tyrosine in addition to 200 μM 6-MePH$_4$, 0.1 μM enzyme, 60 μg/mL catalase, 200 μM NADH, 0.05 unit/mL sheep dihydropteridine reductase, and 80 mM Hepes (pH 7.0) at 30 °C.

Steady-state kinetic data were fit directly to the Michaelis−Menten equation using KaleidaGraph (Synergy) to obtain V_{max}, K_m, and V_{max}/K_m values. The ratio of moles of aromatic amino acid hydroxylated per mole of 6-MePH$_4$ oxidized was obtained by dividing the V_{max} value for amino acid hydroxylation by the V_{max} value for 6-MePH$_4$ oxidation.

RESULTS

Alanine-Scanning Mutagenesis of the Asp425 Loop. The flexible loop that contains Asp425 includes residues 423−427. To learn whether the entire loop is important for tyrosine hydroxylation we carried out alanine scanning of the residues in the loop. The steady-state kinetic parameters of TyrH variants Y423A, Q424A, D425A, Q426A, and T427A are listed in Table 1. TyrH D425A exhibits a 500-fold decrease in tyrosine hydroxylation activity, with the V_{max} and V_{max}/K_{tyr} values exhibiting identical decreases. In contrast, replacing any of the other residues in the loop with alanine has only modest effects on these steady-state kinetic parameters, suggesting that the side chains of Tyr423, Gln424, Gln426, and Thr427 are not critical for the ability of the enzyme to hydroxylate tyrosine. Of the residues in this loop, only Asp425 is critical for DOPA formation.

Table 1. Steady-State Kinetic Parameters for Alanine-Scanning Mutants of TyrH Residues 423−427[a]

enzyme	K_{tyr} (μM)	V_{max} (min^{-1})	V_{max}/K_{tyr} (μM^{-1} min^{-1})
wild-type TyrH	40 ± 4	200 ± 12	5.00 ± 0.25
Y423A	30 ± 6	108 ± 6	3.6 ± 0.7
Q424A	44 ± 5	125 ± 5	2.8 ± 0.3
D425A	56 ± 19	0.54 ± 0.01	0.0097 ± 0.0033
Q426A	66 ± 12	144 ± 12	2.2 ± 0.4
T427A	51 ± 10	174 ± 10	3.4 ± 0.7

[a]Conditions: 0.1 μM enzyme, 10−400 μM tyrosine, 400 μM 6-methyltetrahydropterin, 100 μg/mL catalase, 10 μM ferrous ammonium sulfate, 1 μM dithiothreitol, 50 mM HEPES (pH 7.0), and 30 °C.

Effects of Saturation Mutagenesis of Asp425 on Steady-State Kinetics. To gain further insight into the role of Asp425, we substituted Asp425 of TyrH with every other amino acid but proline. (We did not make the proline variant, because we thought it would cause drastic structural changes in a hairpin loop.) Most of the variants did not express as readily as the wild-type enzyme. For many, the bacterial cells had to be grown at 18 °C to minimize formation of inclusion bodies, and we obtained only ∼2 mg of protein per liter of bacterial culture. Because the previous analysis of the D425V enzyme showed a large change in the preference for phenylalanine versus tyrosine as the amino acid substrate,[16] the kinetics of each variant were characterized with both of these amino acids. This was done both by measuring directly the amount of hydroxylated amino acid product formed and by measuring the rate of tetrahydropterin oxidation in the presence of phenylalanine or tyrosine.

The steady-state kinetic parameters for catalysis of hydroxylation of tyrosine to DOPA by the Asp425 variants are listed in Table 2. Only the D425E and D425Q enzymes produce sufficient DOPA for measurement of the V_{max} and K_{tyr} values using the standard colorimetric assay. For the other variants, we used HPLC separation of the products detected by fluorescence to determine the amount of DOPA produced from tyrosine. For these variants, the K_{tyr} value was determined by measuring the rate of 6-MePH$_4$ oxidation as a function of tyrosine concentration; this was combined with the V_{max} value from HPLC analyses to obtain the V_{max}/K_{tyr} value. For the wild-type enzyme and the D425E and D425Q enzymes, the K_{tyr} values determined from the two assays are identical (results not shown). In the case of TyrH D425W, no DOPA could be detected even with the more sensitive HPLC assay; therefore, we are able to set only an upper limit for the V_{max} value for this variant.

Table 2. Steady-State Kinetic Parameters for Tyrosine Hydroxylation by TyrH Asp425 Variants[a]

enzyme	K_{tyr} (μM)	V_{max} (min^{-1})	V_{max}/K_{tyr} (mM^{-1} min^{-1})
wild-type	40 ± 4	200 ± 12	5000 ± 250
D425A	56 ± 19	0.54 ± 0.01	9.7 ± 3.3
D425C	15 ± 4.3	0.33 ± 0.02	22 ± 6
D425E	97 ± 10	13.4 ± 0.4	140 ± 10
D425F	17.2 ± 4.2	0.12 ± 0.03	7.2 ± 2.7
D425G	10.6 ± 2.8	0.4 ± 0.1	37 ± 13
D425H	18.7 ± 5.7	0.067 ± 0.022	3.6 ± 1.6
D425I	41 ± 8	0.08 ± 0.01	1.9 ± 0.5
D425K	2390 ± 910	0.38 ± 0.07	0.16 ± 0.07
D425L	6.0 ± 1.9	0.042 ± 0.005	7.0 ± 2.4
D425M	6.9 ± 1.6	0.27 ± 0.06	39 ± 13
D425N	172 ± 27	4.6 ± 0.2	26.7 ± 4.4
D425Q	162 ± 32	7.3 ± 0.4	45 ± 7
D425R	88 ± 28	0.018 ± 0.006	0.204 ± 0.093
D425S	8.9 ± 1.6	1.01 ± 0.007	113 ± 20
D425T	1.05 ± 0.22	0.44 ± 0.02	423 ± 91
D425V	40 ± 17	0.038 ± 0.003	0.94 ± 0.40
D425W	nd[b]	<0.008	nd[b]
D425Y	30.8 ± 4.4	0.15 ± 0.02	4.84 ± 0.95
WT PheH[c]		<0.1	0.036 ± 0.003

[a]Conditions as for Table 1. [b]No detectable DOPA formation. [c]From ref 16.

For most of the variants, replacing Asp425 with another amino acid has modest effects on the K_{tyr} value. With the exception of the D425K and D425T enzymes, the change is no more than 4-fold, with both increases and decreases seen. In contrast, the changes in the V_{max} and V_{max}/K_{tyr} values are much greater. In addition, the relative changes in these two parameters are comparable for each variant. This is readily illustrated by plotting V_{max} versus V_{max}/K_{tyr}, as is done in Figure 3A. Here, the data are plotted in logarithmic form to accommodate the large range of the changes, which span 4 orders of magnitude. As might be expected, the most active variant for tyrosine hydroxylation is TyrH D425E, with less than 10% of the activity of the wild-type enzyme. The variants containing amide residues, TyrH D425Q and D425N, have comparable but lower activity, with the V_{max} value being affected less than the V_{max}/K_{tyr} value, while the variants containing alcohol residues, TyrH D425S and D425T, exhibit the opposite pattern, in that the V_{max} value is affected more

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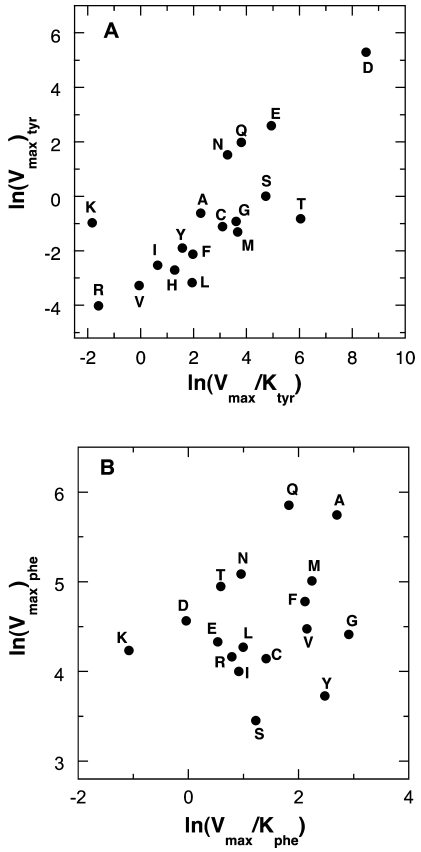

Figure 3. Relationship of V_{max} and V_{max}/K_m values for TyrH Asp425 variants with tyrosine (A) or phenylalanine (B) as the substrate.

than the V_{max}/K_{tyr} value. The variants containing small hydrophobic side chains have less activity, followed by those with large uncharged side chains. The lowest activity is seen with the D425K and D425R enzymes, in which the change from a negatively charged side chain to one that is positively charged is clearly the least conservative change. Even then, the ability of the slowest variant to hydroxylate tyrosine is still greater than that of PheH based on relative V_{max}/K_{tyr} values.

While few of the variants exhibit substantial activity at tyrosine hydroxylation, most of them are quite active at phenylalanine hydroxylation (Table 3). The exception is TyrH D425W, for which we were not able to detect any tyrosine formation using the standard assay. Only the D425H and D425K enzymes have higher K_{phe} values than wild-type TyrH, and we were not able to measure a V_{max} value for the former. All of the variants except TyrH D425H have lower K_{phe} values than wild-type PheH; in general, the variants containing small, uncharged side chains have K_{phe} values more than 10-fold lower than that of wild-type PheH and 5-fold lower than that of wild-type TyrH. There is a reasonable correlation between the K_{phe} and K_{tyr} values for the individual mutants, with an R^2 of 0.90 if the values for the D425K and wild-type enzyme are omitted (results not shown). This correlation is consistent with similar binding of the two amino acid substrates and suggests that the side chain of Asp425 does not interact with the phenolic moiety of tyrosine. Indeed, structures of PheH with amino acids bound show that the corresponding residue in that enzyme, Val379, does not interact directly with the amino acid substrate.[27]

None of the variants has a V_{max} value for phenylalanine hydroxylation as high as that for wild-type PheH, but several

Table 3. Steady-State Kinetic Parameters for Phenylalanine Hydroxylation by TyrH Asp425 Variants[a]

enzyme	K_{phe} (μM)	V_{max} (min^{-1})	V_{max}/K_{phe} (μM^{-1} min^{-1})
wild-type	100 ± 15	96 ± 12	0.96 ± 0.19
D425A	21 ± 3	313 ± 8	14.8 ± 2.1
D425C	15.2 ± 2.0	63 ± 6	4.1 ± 0.2
D425E	45 ± 11	76 ± 13	1.7 ± 0.06
D425F	14.4 ± 4.2	119 ± 22	8.3 ± 2.9
D425G	5.3 ± 1.4	82.6 ± 0.7	18.4 ± 4.9
D425H	>5000	–	0.14 ± 0.02
D425I	22 ± 4	54.6 ± 4.6	2.5 ± 0.5
D425K	200 ± 15	69 ± 10	0.34 ± 0.10
D425L	19 ± 6	71.6 ± 7.3	2.7 ± 0.6
D425M	16.4 ± 0.4	150 ± 16	9.4 ± 1.8
D425N	62 ± 14	162 ± 5	2.6 ± 0.6
D425Q	56.5 ± 9.4	349 ± 16	6.2 ± 1.1
D425R	28 ± 7	64.4 ± 6.4	2.2 ± 0.8
D425S	9.2 ± 4.6	31.6 ± 4.5	3.4 ± 1.3
D425T	16 ± 5	141 ± 2	1.8 ± 0.6
D425V	14.8 ± 2.2	87.8 ± 1.7	8.6 ± 1.4
D425W	nd[c]	nd[c]	nd[c]
D425Y	10.5 ± 2	41.6 ± 1.6	11.9 ± 1.4
WT PheH[b]	252 ± 54	960 ± 41	3.8 ± 0.8

[a]Conditions: 80 mM Hepes (pH 7.0), 0.1 μM enzyme, 5−2000 μM phenylalanine, 200 μM 6-$MePH_4$, 100 μg/mL of catalase, 1 μM dithiothreitol, 10 μM ferrous ammonium sulfate, and 30 °C. [b]From ref 16. [c]No detectable DOPA formation.

variants have significantly higher values than wild-type TyrH. The V_{max} values for phenylalanine hydroxylation vary 25-fold among the variant enzymes with measurable values, substantially less than the 5000-fold range of V_{max} values for tyrosine hydroxylation. Figure 3B shows a plot of the V_{max} values for phenylalanine hydroxylation versus the V_{max}/K_{phe} values. Comparison with Figure 3A illustrates the much larger effects of the mutations on the kinetic parameters with tyrosine as the substrate. In addition, there is much less of a pattern in the effects of the mutations on the phenylalanine hydroxylation kinetics, with the exception that the lowest activity is seen with the positively or negatively charged side chains, especially when the high K_{phe} value of the D425H enzyme is considered.

Effects of Saturation Mutagenesis of Asp425 on Coupling of Amino Acid Hydroxylation and Tetrahydropterin Oxidation. In the chemical mechanism shown in Scheme 2, oxygen, the tetrahydropterin, and the active site iron react to form the Fe(IV)=O hydroxylating species without any reaction involving the amino acid substrate directly,[28,29] although an amino acid must be bound before the active site iron can react with oxygen to form the Fe(IV)=O intermediate.[30,31] For wild-type TyrH, tetrahydropterin oxidation and tyrosine hydroxylation are tightly coupled, so that one molecule of tyrosine is hydroxylated for each molecule of tetrahydropterin oxidized.[2] However, the use of nonphysiological substrates[7] or mutagenesis of TyrH can result in an uncoupling of tetrahydropterin oxidation and amino acid hydroxylation.[14,18,32−34] Consequently, we determined how mutagenesis of Asp425 affected the coupling of tetrahydropterin oxidation and amino acid hydroxylation with both tyrosine and phenylalanine as substrates. This was done by determining the V_{max} value for each mutant using an assay that measured oxidation of 6-$MePH_4$. Dividing the V_{max} value determined by measuring amino acid hydroxylation by the V_{max} value for 6-

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$MePH_4$ oxidation yielded the extent of coupling. These data are listed in Table 4.

Table 4. Moles of Amino Acid Hydroxylated per Mole of Tetrahydropterin Oxidized for TyrH Asp425 Variants[a]

	tyrosine as the substrate		phenylalanine as the substrate	
enzyme	V_{max} (min^{-1}), 6-$MePH_4$ oxidation	coupling (%)	V_{max} (min^{-1}), 6-$MePH_4$ oxidation	coupling (%)
TyrH	239 ± 17	84 ± 9	349 ± 22	28 ± 4
D425A	71 ± 9	0.76 ± 0.22	715 ± 20	44 ± 1
D425C	30.2 ± 1.6	1.1 ± 0.1	100 ± 3	63 ± 7
D425E	119 ± 12	11.0 ± 0.6	125 ± 5	61 ± 12
D425F	48.2 ± 2.8	0.26 ± 0.09	118 ± 9	101 ± 27
D425G	37 ± 2	1.1 ± 0.3	210 ± 8.4	39 ± 2
D425H	48 ± 7	0.14 ± 0.05	107 ± 2	nd[b]
D425I	52 ± 12	0.15 ± 0.03	111 ± 8	49 ± 5
D425K	42 ± 9	0.46 ± 0.18	115 ± 5	60 ± 10
D425L	31.5 ± 1.4	0.13 ± 0.05	192 ± 11	37 ± 4
D425M	159 ± 6	0.17 ± 0.04	279 ± 11	54 ± 6
D425N	117 ± 6	3.9 ± 0.4	266 ± 10	61 ± 2
D425Q	214 ± 13	3.40 ± 0.03	358 ± 17	98 ± 6
D425R	19.6 ± 1.3	0.092 ± 0.058	96 ± 71	67 ± 8
D425S	91.7 ± 2.5	1.1 ± 0.04	89.4 ± 3.5	35 ± 5
D425T	44 ± 1	1.00 ± 0.07	221 ± 12	64 ± 4
D425V	46.6 ± 7.1	0.081 ± 0.007	214 ± 1	41 ± 1
D425W	3.2 ± 0.5	nd[b]	1.6 ± 0.3	nd[b]
D425Y	105 ± 4	0.14 ± 0.02	31.5 ± 1.8	132 ± 9

[a]Calculated from V_{max} values for tetrahydropterin oxidation and V_{max} values for amino acid hydroxylation. [b]None detected.

All of the variants oxidize 6-$MePH_4$ in the presence of tyrosine, including TyrH D425W, for which we were unable to detect any DOPA formation, but none are as active at tyrosine-dependent 6-$MePH_4$ oxidation as the wild-type enzyme. In addition, all of the mutations significantly uncouple 6-$MePH_4$ oxidation and tyrosine hydroxylation. Even for the most active variant, TyrH D425E, only 11% of the oxidizing equivalents from the tetrahydropterin are consumed productively. For a number of the variants, the level of uncoupling is greater than 99%, with TyrH D425V being the most uncoupled.

All of the variants also oxidize 6-$MePH_4$ in the presence of phenylalanine. Again, TyrH D425W has detectable but very low activity as a phenylalanine-dependent tetrahydropterin oxidase. For all of the variants except TyrH D425Y and even for wild-type TyrH, the V_{max} value for 6-$MePH_4$ oxidation with phenylalanine is equal to or greater than that with tyrosine as the amino acid substrate. The phenylalanine-dependent 6-$MePH_4$ oxidation activity with TyrH D425A is twice that of the wild-type enzyme and approaches the V_{max} value for phenylalanine hydroxylation by liver PheH (Table 3). The extent of uncoupling of 6-$MePH_4$ oxidation and phenylalanine hydroxylation for the variants is much lower than for tyrosine hydroxylation. Indeed, in no case does mutagenesis of Asp425 decrease the level of coupling of phenylalanine hydroxylation and 6-$MePH_4$ oxidation below what is seen with wild-type TyrH, and for the D425F, D425Q, and D425Y enzymes, the reactions are completely coupled, unlike that of the wild-type enzyme.

The magnitude (up to 10^4) of the changes in the extent of coupling of 6-$MePH_4$ in the presence of tyrosine for the different variants resembles the large changes seen in the V_{max} value for formation of DOPA from tyrosine. Figure 4 shows a plot of the relationship between these two parameters for both amino acid substrates. There is clearly a direct relationship between the ability of the variants to convert tyrosine to DOPA at saturating concentrations of the amino acid and the extent to which tetrahydropterin oxidation is coupled to DOPA formation, establishing that most of the loss of the ability to hydroxylate tyrosine is due to uncoupling of tetrahydropterin oxidation and DOPA formation. In contrast, the ability of TyrH to catalyze hydroxylation of phenylalanine and the coupling of tetrahydropterin oxidation to this reaction are both far less sensitive to mutagenesis of Asp425.

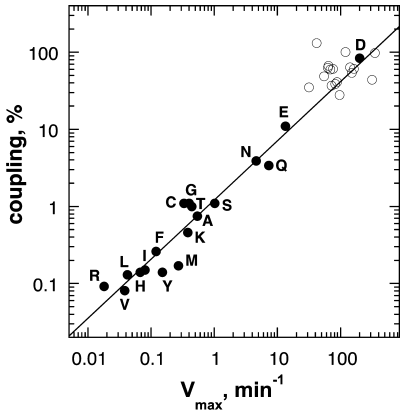

Figure 4. Correlation between the V_{max} values for hydroxylation of tyrosine (●) or phenylalanine (○) and the extent of coupling of 6-$MePH_4$ oxidation and amino acid hydroxylation for TyrH Asp425 variants.

Structure–Function Analyses. To gain insight into the basis for the effects of substitution of Asp425 with other amino acid residues, we examined whether there are any correlations between the individual kinetic parameters given in Tables 2–4 and physical properties of the individual amino acid residues. For the K_m and V_{max}/K_m values with either tyrosine or phenylalanine as the substrate, there is no significant correlation with the volume,[35,36] accessible surface area,[37] or any of several measures of the hydrophobicity[38−42] of the individual residues (results not shown). In contrast, there is a strong negative correlation between the V_{max} value for tyrosine hydroxylation and the hydrophobicity of the amino acid residue at position 425 (Table S1 of the Supporting Information). If the charged amino acid residues are omitted from the analysis, the best quantitative correlation is with the free energy of partitioning of the individual residues between water and cyclohexane as reported by Radzicka and Wolfenden.[41] As shown in Figure 5, the V_{max} value for tyrosine hydroxylation decreases as the hydrophobicity of the residue at that position increases. The extent of coupling shows a similar correlation (Table S1 of the Supporting Information), not surprisingly. In contrast, for tyrosine formation from phenylalanine, the V_{max} value is affected little by the hydrophobicity of the residue at position 425. The extent of coupling with phenylalanine as the substrate does correlate with the accessible surface area of the residue, regardless of whether the charged amino acid residues are considered, but the effects are much smaller than when tyrosine is the amino acid substrate (Table S1 and Figure S1 of the Supporting Information).

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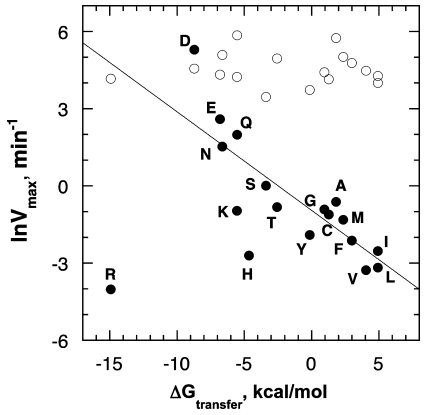

Figure 5. Correlation of V_{max} values for hydroxylation of tyrosine (●) or phenylalanine (○) by TyrH Asp425 variants with the free energy of transfer from cyclohexane to water of the amino acid residue at position 425. The line is a linear fit of the ln(V_{max}) values for tyrosine hydroxylation, omitting the values for wild-type TyrH and the D425E, D425H, D425K, and D425R variants.

The substrate specificity of the different variants, as reflected in the ratio of the V_{max}/K_{tyr} value to the V_{max}/K_{phe} value, shows a weak negative correlation with the hydrophobicity of the residue, with an R^2 value of 0.40 versus the energy of transfer from cyclohexane to water when the charged residues are not included and an R^2 value of 0.27 versus the estimated hydrophobic effect for side chain burial from Karplus[42] when charged residues are also considered (Table S1 of the Supporting Information). The wild-type enzyme has the highest specificity for tyrosine over phenylalanine, while TyrH D425V has the highest specificity for phenylalanine over tyrosine.

■ DISCUSSION

The kinetic analyses of the TyrH Asp425 variants in Tables 2−4 establish that this residue plays a critical role in the ability of TyrH to catalyze the hydroxylation of tyrosine to DOPA, as reflected in the large decreases in the V_{max} and V_{max}/K_{tyr} values for DOPA formation. The predominant effect of mutating Asp425 is to decrease the efficiency with which oxidation of the tetrahydropterin is coupled to hydroxylation of tyrosine to form DOPA. In contrast, almost all of the mutations make the enzyme a better phenylalanine hydroxylase, irrespective of whether one uses K_{phe}, V_{max}, V_{max}/K_{phe}, or the efficiency of coupling of 6-MePH$_4$ oxidation to phenylalanine hydroxylation as a criterion.

Hydroxylation of phenylalanine by PheH results in tyrosine as the sole amino acid product, but both tyrosine and 3-hydroxyphenylalanine are produced when phenylalanine is the substrate for TyrH, at a ratio of 25:1.[43] Similarly, when 4-CH$_3$-phenylalanine is the substrate for PheH, the products 4-HOCH$_2$-phenylalanine and 4-HO,3-CH$_3$-phenylalanine arise from addition of oxygen at carbon 4 or the methyl group,[44] whereas the additional product 3-HO,4-CH$_3$-phenylalanine is produced from 4-CH$_3$-phenylalanine by TyrH by hydroxylation at carbon 3.[7,45] Tyrosine can bind to PheH, leading to a reaction with oxygen in which the oxygen−oxygen bond is broken to form the 4a-hydroxypterin product,[46] but no DOPA is produced. This is consistent with formation of the Fe(IV)═O intermediate with tyrosine as the substrate, but an inability of the Fe(IV)═O intermediate to hydroxylate the aromatic ring of the amino acid substrate when it already has one hydroxyl. These results suggest that the difference in specificity between PheH and TyrH is not an intrinsic preference for hydroxylation at different carbons on the side chain of the aromatic amino acid substrate. Instead, it is that the active site of PheH has evolved so that only a single carbon on the amino acid substrate is accessible to the Fe(IV)═O intermediate. That the position at which this single oxygen is added can be malleable is demonstrated by the results of Zhang et al. with the PheH PacX.[47] This enzyme hydroxylates phenylalanine at position 3 of the aromatic ring rather than at position 4. Upon mutagenesis of Cys187 and Thr202, residues in the hydrophobic cage surrounding the amino acid substrate, to the residues found in eukaryotic forms of PheH, the product of phenylalanine hydroxylation changed from only 3-hydroxyphenylalanine to >92% tyrosine, consistent with steric factors determining where the hydroxylating intermediate in PheH attacks the aromatic ring of the amino acid substrate.

Soybean lipoxygenase-1 (SLO) provides another example of an enzyme that controls the site of oxygen reactivity with optimal placement of hydrophobic residues. In that enzyme, mutagenesis of two leucines to smaller alanines to open access to carbon 9 of the substrate linoleic acid led to reaction of oxygen at carbon 9 in addition to carbon 13.[48]

The intrinsic preference of TyrH to form tyrosine from phenylalanine and the ability of the enzyme to add an oxygen at carbon 3 suggest that the evolutionary change(s) that converted PheH to TyrH predominantly opened the active site rather than simply changed the site of oxygen addition by PheH. New enzymes are generally thought to arise by gene duplication and then divergence from a preexisting enzyme that catalyzes a related reaction and already has the required new activity at a low level.[49−52] Thus, TyrH would have diverged from the ancestral PheH by acquiring amino acid changes that enhanced its ability to hydroxylate tyrosine. The results presented here suggest that a critical change that allowed this to occur was the introduction of an aspartate residue at position 425. This mutation could have involved a simple one-base change, from a GTC for valine in the PheH enzymes to a GAC in the TyrH enzymes. (The homologous position in TrpH has an isoleucine residue, and the codon used is ATC.) However, while the evolution of a new enzyme activity typically results in a decrease or even loss of the original activity,[52] in the case of TyrH the underlying ability to hydroxylate phenylalanine was not lost and is indeed quite robust in that phenylalanine hydroxylation by TyrH is much less sensitive to mutagenesis than is tyrosine hydroxylation. Residual PheH activity in TyrH would not necessarily be deleterious and thus would not necessarily have been eliminated by evolutionary pressure. The activity of TyrH is heavily regulated,[53] such that it is essentially inactive unless catecholamine synthesis is required. A background activity of phenylalanine hydroxylation to make more tyrosine at the same time tyrosine was being converted to DOPA would even have some advantage in maintaining tyrosine levels. In contrast, PheH is an allosteric enzyme, so that there is always some level of activity.[54] Formation of the reactive DOPA from tyrosine in the liver cytosol, where it could not be metabolized further, would be damaging to the cell. This would likely provide sufficient evolutionary pressure to yield a PheH unable to hydroxylate tyrosine.

The correlation of the decrease in the efficiency of coupling tetrahydropterin oxidation and DOPA formation with the hydrophobicity of the residue at position 425 (Figure 5) is striking. A large number of hydrophobicity scales for amino

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acid residues have been developed over the years, primarily to gain insight into the contribution of hydrophobic interactions to protein folding.[55,56] The accuracy with which any specific hydrophobicity scale predicts the effects of site-directed mutagenesis on protein structure is clearly context-dependent, and charged and polar amino acid side chains are capable of interactions other than the hydrophobic kind.[42] In light of these complications, we did not attempt to determine the hydrophobicity scale that gave the best fit to the data from the many available scales but instead focused on a representative selection of hydrophobicity scales and other properties of amino acid side chains. The negative correlation of the coupling with the free energy of transfer from water to cyclohexane suggests that a hydrophobic interaction involving the residue at position 425 results in a restricted active site in which hydroxylation of tyrosine is not possible. This is consistent with the finding that TyrH D425V, the least coupled variant other than TyrH D425W, contains the residue found at this position in PheH, an enzyme that is essentially unable to catalyze tyrosine hydroxylation.

The hydrophobic interaction is likely with Phe184, which is phenylalanine or tyrosine in all available sequences of TyrH. This residue lies on a dynamic loop that extends from His178 to Asp189; much of this loop is not seen in the available structures of TyrH in the presence or absence of bound pterin,[9,57] and there is no structure of TyrH with an amino acid bound. However, crystal structures of PheH do show that the homologous loop in that protein, Ala132−Asp143, closes down upon the active site when both the amino acid substrate and tetrahydrobiopterin are bound, so that the side chain of Val379 is 3.7 Å from the face of the aromatic ring of Tyr138 (Figure 6).[58] Fluorescence studies of TyrH have established that this loop also closes down in response to substrate binding to that enzyme,[59] and alanine-scanning mutagenesis of residues in the center of the loop in TyrH uncouples tetrahydropterin oxidation and DOPA formation, with the F184A mutation having the greatest effect.[18] The importance of the interaction between PheH Val379 and Tyr138 in determining substrate specificity is supported by studies of the PheH Y138F/V379D double mutant.[18] Substitution with the two residues found at these positions in TyrH results in a PheH with substantial activity at tyrosine hydroxylation and an 800-fold decrease in the specificity for phenylalanine versus tyrosine. The correlation in Figure 5 between the V_{max} values for tyrosine hydroxylation for the Asp425 mutants and the free energy for transfer of the side chain into cyclohexane suggests that a primary role of Asp425 is to disrupt the hydrophobic interaction with Phe184, yielding a more open active site that allows hydroxylation of tyrosine.

Alternate roles for Asp425 can be ruled out by these data. Asp425 is not needed to stabilize the cationic intermediate formed during electrophilic aromatic substitution, because the PheH and TrpH reactions also involve the same intermediate (Scheme 2). Asp425 is not involved in hydrogen bonding to the OH group of tyrosine; if it were, we would see a very different K_{tyr} value upon substitution of Asp425 with any of the hydrophobic residues, and this is not the case.

These results suggest that the change from valine to aspartate at position 425 was critical in the evolution of the enzyme from a phenylalanine hydroxylase to a tyrosine hydroxylase. Subsequent mutations in TyrH have not suppressed the intrinsic ability of the enzyme to hydroxylate phenylalanine, suggesting that there has been a lack of evolutionary pressure to eliminate that activity.

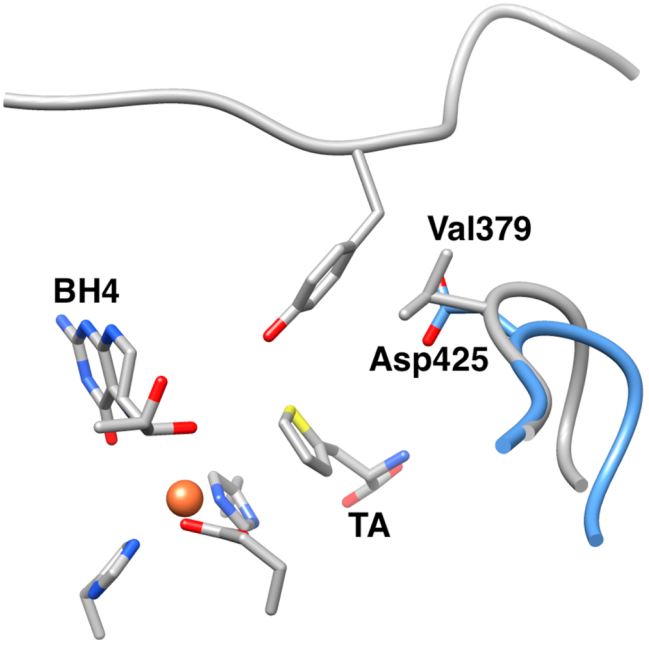

Figure 6. Comparison of the positions of Asp425 in TyrH and Val379 in PheH showing the interaction between Val379 and Tyr138 in PheH with both tetrahydrobiopterin (BH4) and an amino acid substrate (thienylalanine, TA) bound. The figure is based on PDB entries 1KW0 and 1TOH.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*S.C.D.: e-mail, sdaubner@stmarytx.edu. P.F.F.: Department of Biochemistry, 7703 Floyd Curl Dr., San Antonio, TX 78229; phone, (210) 567-8264; e-mail, fitzpatrick@biochem.uthscsa.edu.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

TyrH, tyrosine hydroxylase; PheH, phenylalanine hydroxylase; TrpH, tryptophan hydroxylase; 6-MePH$_4$, 6-methyltetrahydropterin; DOPA, 3,4-dihydroxyphenylalanine; IPTG, isopropyl β-thiogalactopyranoside; Protein Data Bank, Protein Data Bank.

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