Reversing the Substrate Specificities of Phenylalanine and Tyrosine Hydroxylase: Aspartate 425 of Tyrosine Hydroxylase Is Essential for L-DOPA Formation†

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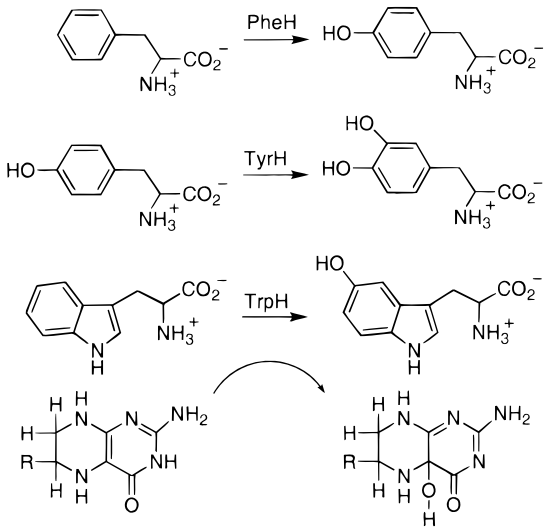
ABSTRACT: The catalytic domains of the pterin-dependent enzymes phenylalanine hydroxylase and tyrosine hydroxylase are homologous, yet differ in their substrate specificities. To probe the structural basis for the differences in specificity, seven residues in the active site of phenylalanine hydroxylase whose side chains are dissimilar in the two enzymes were mutated to the corresponding residues in tyrosine hydroxylase. Analysis of the effects of the mutations on the isolated catalytic domain of phenylalanine hydroxylase identified three residues that contribute to the ability to hydroxylate tyrosine, His264, Tyr277, and Val379. These mutations were incorporated into full-length phenylalanine hydroxylase and the complementary mutations into tyrosine hydroxylase. The steady-state kinetic parameters of the mutated enzymes showed that the identity of the residue in tyrosine hydroxylase at the position corresponding to position 379 of phenylalanine hydroxylase is critical for dihydroxyphenylalanine formation. The relative specificity of tyrosine hydroxylase for phenylalanine versus tyrosine, as measured by the $(V/K_{phe})/(V/K_{tyr})$ value, increased by 80000-fold in the D425V enzyme. However, mutation of the corresponding valine 379 of phenylalanine hydroxylase to aspartate was not sufficient to allow phenylalanine hydroxylase to form dihydroxyphenylalanine at rates comparable to that of tyrosine hydroxylase. The double mutant V379D/H264Q PheH was the most active at tyrosine hydroxylation, showing a 3000-fold decrease in the $(V/K_{phe})/(V/K_{tyr})$ value.

The tetrahydropterin-dependent aromatic amino acid hydroxylases phenylalanine hydroxylase (PheH),[1] tyrosine hydroxylase (TyrH), and tryptophan hydroxylase (TrpH) constitute a family of enzymes which are closely related in structure and mechanism (*1*, *2*). The reactions these enzymes catalyze in vivo appear in Scheme 1. All three enzymes are rate-limiting for important metabolic pathways: PheH, phenylalanine catabolism; TyrH, catecholamine synthesis; and TrpH, serotonin synthesis. Deficiencies in these enzymes contribute to a number of disease states in humans. Low levels of PheH result in phenylketonuria (*3*), and aberrant levels of TyrH and TrpH are factors in a variety of nervous system diseases (*4*−*9*).

Each of these enzymes contains iron, utilizes tetrahydropterins, and hydroxylates an aromatic amino acid using molecular oxygen. These enzymes display a remarkable degree of sequence similarity in their 335 C-terminal amino acid residues, where PheH and TyrH are 75% identical and all three enzymes are 50% identical (*10*). Figure 1 shows an alignment of the sequences of rat PheH and rat TyrH. Deletion mutagenesis and proteolysis of the native enzymes have shown that the 335 C-terminal amino acids contain the residues responsible for catalysis (*11*−*15*). The amino termini of the three enzymes differ greatly, and are assumed to play regulatory roles in each enzyme (*2*). It has been suggested that the catalytic (C) domains of the three hydroxylases evolved separately from the regulatory (R) domains, and that

Scheme 1



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[1] Abbreviations: DOPA, dihydroxyphenylalanine; TyrH, tyrosine hydroxylase; PheH, phenylalanine hydroxylase; TrpH, tryptophan hydroxylase; tPH, catalytic domain of rat phenylalanine hydroxylase; nT-PAH, chimeric protein with the regulatory domain of TyrH and the catalytic domain of PheH; 6-MPH_4, 6-methyltetrahydropterin; BH_4, tetrahydrobiopterin; K_{tyr}, K_M value for tyrosine; K_{phe}, K_M value for phenylalanine; LB, Luria-Bertani medium.

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TyrH    1 MATPSAPSPQPKGFRRAVSEQDAKQAEAVTSPRFIGRRQSLIEDARKEREAAAAAAAAAVASSEPGNPLEAVVFE  75
PheH    1 MAAVVLENG---VLSRKLSD--------------FGQETSYIEDN---------------S-NQ-----------  31

TyrH   76 ERDGNAVLNLLFSLRGTKPSSLSRAVKVFETFEAKIHHLETRPAQRPLAGSPHLEYFVRFEVPSGDLAALLSSVR 150
PheH   32 ----NGAISLIFSLKEEVG-ALAKVLRLFEENDINLTHIESRPSRLNKDEYEFFTYLDKRTKP--VLGSIIKSLR  99

TyrH  151 -RVSDDVRSAREDK----VPWFPRKVSELDKCHHLVTKFDPDLDLDHPGFSDQVYRQRRKLIAEIAFQYKHGEPI 220
PheH  100 NDIGATVHELSRDKEKNTVPWFPRTIQELDRFANQILSYGAELDADHPGFKDPVYRARRKQFADIAYNYRHGQPI 174

TyrH  221 PHVEYTAEEIATWKEVYVTLKGLYATHACREHLEGFQLLERYCGYREDSIPQLEDVSRFLKERTGFQLRPVAGLL 295
PheH  175 PRVEYTEEEKQTWGTVFRTLKALYKTHACYEHNHIFPLLEKYCGFREDNIPQLEDVSQFLQTCTGFRLRPVAGLL 249

TyrH  296 SARDFLASLAFRVF Q CTQYIRHASSPM H SPEPDCCHELLGHVPMLADRTFAQFSQDIGLASLGASDEEIE 365
PheH  250 SSRDFLGGLAFRVF H CTQYIRHGSKPM Y TPEPDICHELLGHVPLFSDRSFAQFSQEIGLASLGAPDEYIE 319

TyrH  366 KLSTVYWFTVEFGLCKQNGELKAYGAGLLSSYGELLHSLSEEPEVRAFDPDTAAVQPYQ D QTYQPVYFVSESF 438
PheH  320 KLATIYWFTVEFGLCKEGDSIKAYGAGLLSSFGELQYCLSDKPKLLPLELEKTACQEYS V TEFQPLYYVAESF 392

TyrH  439 NDAKDKLRNYASRIQRPFSVKFDPYTLAIDVLDSPHTIQRSLEGVQDELHTLAHALSAIS   498
PheH  393 SDAKEKVRTFAATIPRPFSVRYDPYTQRVEVLDNTQQLKILADSINSEVGILCNALQKIKS  453
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FIGURE 1: Alignment of the amino acid sequences of rat TyrH and rat PheH. The alignment was generated by the program MacVector from Oxford Molecular. The first sequence in each line is that of TyrH and the second PheH. Similar amino acids are indicated by underlining and identical amino acids in bold. The residues which gave PheH the greatest ability to hydroxylate tyrosine are denoted by much larger type.

the DNA sequences encoding the R domains were conscripted from different sources (*16*). Each enzyme also has a tetramerization domain located at the very carboxyl end of the protein, which utilizes a repeating sequence of hydrophobic residues to polymerize via coiled-coil interactions (*15*, *17*).

The three-dimensional structures of the catalytic domains of TyrH (*18*, *19*) and PheH (*20*, *21*) have recently been determined. The two enzymes have very similar active sites. Each consists of a cleft formed by four α-helices, about 17 Å deep and 15 Å wide, with an iron atom 10 Å into the cleft. In each enzyme, the protein ligands to the iron are two histidine residues and a glutamate residue. Several other active site residues are also conserved in both enzymes; analyses of these residues in rat TyrH have shown that Arg316 and Asp328 are involved in binding tyrosine and Glu332 and Phe300 are involved in binding tetrahydropterin (*22*, *23*). These residues correspond to Arg270, Asp282, Glu286, and Phe254 in rat PheH.

In contrast to their striking similarities, the enzymes also exhibit some important differences. As is clear in Figure 1, the R domains of TyrH and PheH differ in sequence and in length. Both enzymes are activated by phosphorylation of serine residues in the respective R domains (*24*, *25*), but the mechanisms of activation differ. In TyrH, phosphorylation results in the alleviation of feedback inhibition by catecholamines (*26*, *27*); in PheH, phosphorylation modulates the degree of activation by phenylalanine and tetrahydrobiopterin (*28*−*30*). The enzymes also have different substrate specificities. PheH is very specific for phenylalanine; although it can hydroxylate tryptophan to a small extent, it is unable to hydroxylate tyrosine (*31*, *32*). TyrH is able to hydroxylate phenylalanine with approximately 66% of the V_{max} value of tyrosine (*33*, *34*). These substrate specificities are determined by the C domains; chimeric and truncated forms of the PheH and TyrH display qualitatively the same amino acid substrate specificity as the full-length enzymes (*35*).

To discover how these two very similar proteins discriminate between very similar substrates, we analyzed the amino acid sequences of TyrH and PheH for active site residues which differ between the two enzymes but are conserved for a specific enzyme. With these criteria, we identified seven residues as potential specificity determinants. The residues in rat PheH (and the corresponding residue in TyrH) are Ser251 (Ala), His264 (Gln), Tyr277 (His), Leu293 (Met), Ala322 (Ser), Tyr356 (His), and Val379 (Asp). Mutant forms of PheH containing the residues found in TyrH and mutant forms of TyrH containing the residues found in PheH were constructed and analyzed. The results show that Asp425 of TyrH is critical for determining the relative specificities of these two enzymes for phenylalanine and tyrosine.

EXPERIMENTAL PROCEDURES

Materials. Custom oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer by the Gene Technology Laboratory of Texas A&M University. Restriction endonucleases were from New England Biolabs Inc. *Pfu* DNA polymerase was obtained from Stratagene USA. Plasmids were purified using kits from Qiagen Inc. (6*R*)-BH_4 and 6-methyltetrahydropterin (6-MPH_4) were purchased from B. Schircks Laboratories. Leupeptin, pepstatin, and catalase were obtained from Boehringer Mannheim Corp. Sheep dihydropteridine reductase, soybean lysophosphatidylcholine, L-tyrosine, L-phenylalanine, and NADH were from Sigma Corp. Heparin-Sepharose and phenyl-Sepharose Fast Flow were purchased from Pharmacia Biotech Inc. 3,5-[^{3}H]Tyrosine was from Amersham Corp.

Vectors for Enzyme Expression. The Stratagene QuikChange method using *Pfu* DNA polymerase was used for mutagenesis. Plasmids pETYH8 and pERPH5 have been previously described (*35*) and were used as the templates for intact rat TyrH and rat PheH, respectively. Plasmid pPCTN2 (*35*) encodes a chimeric mutant hydroxylase (nT-PheH) consisting of the C domain of PheH and the R domain of TyrH; it was the template for mutagenesis of the chimeric enzyme. Plasmid pERPHΔ117 (*35*) encodes expression of the C domain of rat phenylalanine hydroxylase (tPH); it was the template for mutagenesis of the isolated domain. The sequences of the oligonucleotides used to direct mutagenesis

Table 1: Sequences of Oligonucleotides Used To Introduce Mutations

enzyme	residue	sequence of the oligonucleotide	restriction enzyme for screening
phenylalanine hydroxylase	S251A	5′-GGCTT ACTGT CAGCA CGAGA TTTCT TGG-3′	*Xho*I
	H264Q	5′-CTTCC GAGTC TTCCA GTGCA CACAG TAC-3′	*Apa*LI
	Y277H	5′-CGAAG CCCAT GCATA CACCT GAACC-3′	*Bsr*GI
	L293M	5′-GGGAC ATGTG CCTAT GTTTT CAGAT CGC-3′	sequence
	A322S	5′-CATTG AGAAA CTGTC GACAA TTTAC TGG-3′	*Hin*cII
	Y356H	5′-GGAGA ATTAC AGCAC TGTTT ATCAG AC-3′	*Sca*I
	V379D	5′-CCAGG AGTAC TCTGA TACAG AGTTC CAGC-3′	*Sca*I
tyrosine hydroxylase	D425V	5′-GCCCT ACCAG GTTCA AACCT ACCAG C-3′	sequence
	Q310H	5′-CCGCG TGTTT CATTG CACCC AG-3′	*Bsr*DI
	H323Y	5′-CCTCA CCTAT GTACA GTCCT GAGCC GG-3′	*Bsr*GI

are listed in Table 1 along with the restriction enzymes used to detect each mutation, when applicable. Mutated plasmids were detected by electrophoretic analysis of restriction digests of plasmids, or, where indicated in Table 1, by DNA sequencing. In all cases, the entire coding regions were sequenced to confirm the desired mutations and to detect unexpected mutations.[2]

Bacterial Cell Growth and Protein Purification. Bacterial cells expressing wild-type rat TyrH and rat PheH were grown as previously described (*35*). To obtain the altered proteins, the mutated plasmids were introduced into competent *Escherichia coli* BL21(DE3) cells. Small cultures of each were stored as 15% glycerol stocks at −80 °C. Cells from permanent stocks containing the appropriate plasmid were streaked on LB agar plates containing 100 μg/mL ampicillin; a single colony from each plate was used to inoculate a 60 mL liquid culture. After incubation of the latter overnight at 37 °C, 10−20 mL was used to inoculate 1 L cultures of LB broth containing 100 μg/mL ampicillin. If the protein being expressed contained the R domain of TyrH, the culture remained at 37 °C for the entire growth. If the protein being expressed contained the R domain of PheH, the culture remained at 37 °C until the A_{600} value reached 0.5, after which the flasks were swirled for 1 min in ice−water and then moved to a 30 °C incubator. For all cultures, when the A_{600} value reached 0.8, isopropyl β-D-thioglucanopyranoside was added to a final concentration of 0.5 mM. After 3 h for cultures growing at 37 °C and 5 h for those growing at 30 °C, cells were harvested by centrifugation at 5000*g* for 30 min and stored at −70 °C overnight.

Purification of wild-type TyrH, mutants of TyrH, chimeric mutant nT-PheH, and active site mutants of nT-PheH was performed as previously described for wild-type TyrH (*26*), except that nucleic acids were precipitated with 2.0% streptomycin sulfate (w/v) prior to ammonium sulfate precipitation. The enzymes were eluted from heparin-Sepharose by a linear gradient from 0 to 800 mM sodium chloride (250 mL total volume) in 50 mM Tris-HCl, 10% glycerol, 75 μM EDTA, 1 μM pepstatin, and 1 μM leupeptin (pH 7.0). For storage, the enzymes were concentrated to approximately 75 μM using Millipore Ultrafree 15 centrifugal concentration devices.

Purification of wild-type PheH and active site mutants of PheH was performed as described by Shiman (*38*) as modified for bacterial sources (*35*). The enzymes were eluted from phenyl-Sepharose in 50 mM Tris-HCl, 15% glycerol, 75 μM EDTA, 1 μM pepstatin, 1 μM leupeptin, and 0.035% Tween 80 (pH 7.0). The enzymes were concentrated using an Amicon ultrafiltration concentrator with PM-30 membranes to approximately 75 μM.

Truncated mutants of PheH (tPH) were not purified; they were assayed in centrifuged extracts so multiple mutations could be screened quickly. For each mutant protein, a single colony was transferred to 30 mL of LB-amp and grown at 37 °C until the A_{600} value of the culture reached 0.8. Isopropyl β-D-thioglucanopyranoside was then added to a final concentration of 0.5 mM. After a further 3 h, cells were harvested by centrifugation at 5000*g* for 30 min. The resulting pellets were resuspended in an 8-fold excess (with respect to cell weight) of 50 mM Tris-HCl, 10% glycerol, 75 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, 50 μg/mL hen egg white lysozyme, and 100 μg/mL phenylmethanesulfonyl fluoride (pH 7.0). The resuspended mixtures were sonicated on ice for six bursts of 15 s with 2 min rests for cooling. The lysates were centrifuged for 20 min at 15000*g* and 4 °C to obtain the centrifuged lysates used for analysis.

Enzyme Assays. Concentrations of tyrosine, phenylalanine, tryptophan, NADH, 6-MPH_4, and BH_4 in stock solutions were determined by UV−visible absorbance measurements on a Hewlett-Packard 8452 diode array spectrophotometer. Changes in absorbance with time during assays were monitored on a Beckman DU640 spectrophotometer. Concentrations of phenylalanine hydroxylases were determined using extinction coefficients calculated from the amino acid compositions by the method of Pace et al. (*39*). Concentrations of wild-type and mutant tyrosine hydroxylases were determined using an $A^{280}_{1\%}$ value of 10.4 (*40*). Steady-state kinetic data were fit directly to the Michaelis−Menten equation using the program Kaleidagraph (Synergy Software).

A colorimetric assay for DOPA (*35*) was used routinely to assess tyrosine hydroxylation. A radiometric assay for tritium release from 3,5-[^{3}H]tyrosine (*41*) was used when measurements were extended to tyrosine concentrations of >1 mM. Conditions for both assays were 50 mM HEPES/NaOH, 100 μg/mL catalase, 10 μM ferrous ammonium

[2] Using the ABI BigDye sequencing kit, a deviation from the published sequence of the cDNA for rat phenylalanine hydroxylase was noted. The published sequence for the residue at position 87 is ACG, the codon for threonine (*36*). Our results consistently showed a sequence of AGC, which encodes serine. Our source plasmid for the coding region of pERPH5, pRPH1, was a gift from I. Jennings of the Royal Children's Hospital (Victoria, Australia). We sequenced pRPH1 repeatedly and found the same codon for serine. These results agree with the findings of Kappock et al. (*37*). Because the published sequences for mouse and human phenylalanine hydroxylase both contain a serine at position 87, it seems probable that the earlier sequence with threonine at position 87 is in error.

sulfate, and 1 mM DTT (pH 6.7 and 32 °C); the concentrations of tyrosine and tetrahydropterins that were used are given in the table footnotes. Assays were carried out for 2−5 min. Enzymes were incubated beforehand for 5 min at 25 °C with and without 1.25 mM lysophosphatidylcholine. Assays were initiated by the addition of enzyme. For determination of steady-state kinetic parameters, 15 assays with varying tyrosine concentrations, bracketing the K_{tyr} value if possible, were used.

Rates of formation of tyrosine from phenylalanine for all enzymes were measured as described previously (*35*, *42*), monitoring the increase in absorbance at 275 nm. The conditions were 50 mM HEPES/NaOH, 50 μg/mL catalase, 10 μM ferrous ammonium sulfate, and 1 mM DTT (pH 6.7 and 32 °C); the concentrations of phenylalanine and tetrahydropterin that were used are given in the table footnotes. For determination of steady-state kinetic parameters, 15 assays with phenylalanine concentrations bracketing the K_{phe} value were used. Phenylalanine hydroxylases were preincubated for 5 min at 25 °C with a 25-fold molar excess of phenylalanine. Tyrosine hydroxylases were incubated without phenylalanine at 25 °C for 5 min prior to the assay. Assays were initiated by the addition of enzyme.

With enzymes which exhibited very low rates of DOPA formation (wild-type and mutant PheH enzymes and D425V TyrH), K_{tyr} values were determined by measuring the rate of tetrahydropterin oxidation as a function of tyrosine concentration. A coupled assay with dihydropteridine reductase was used, monitoring the decrease in absorbance at 340 nm due to NADH oxidation. The background rate due to autoxidation of the tetrahydropterin was subtracted when calculating the rate of the enzymatic reaction. The conditions were 50 mM HEPES/NaOH, 100 μg/mL catalase, 340 μM 6-MPH_4, 200 μM NADH, 10 μM ferrous ammonium sulfate, and 0.2 unit/mL sheep dihydropteridine reductase (pH 6.7 and 32 °C). Assays were initiated by the addition of enzyme which had been incubated for 5 min at 25 °C with 1.25 mM lysophosphatidylcholine.

RESULTS

Activity of Truncated PheH Mutants. Since PheH is reported to be unable to form DOPA (*43*), we first screened for mutated phenylalanine hydroxylases which could form DOPA. For initial screening for tyrosine hydroxylation by PheH, site-specific mutants of the isolated C domain of PheH (tPH) were designed. The enzyme tPH is overexpressed in much larger quantities than full-length PheH, and has the same substrate specificity. In addition, truncated PheH does not require preincubation with phenylalanine or lipids for full activity (*35*). Centrifuged lysates of cells expressing tPH proteins mutated at the sites listed in Table 1 were assayed for the ability to catalyze DOPA formation. The resulting data are given in Table 2. Denaturing polyacrylamide gels showed comparable expression of all the proteins (data not shown), so differences among them are not due to expression level. The data shown were collected using BH_4; similar results were obtained using 6-MPH_4, although the activities were about 2-fold lower in that case (data not shown). The first eight enzymes in Table 2 were constructed, expressed, and analyzed initially. Any protein of this series which contained V379D was able to hydroxylate tyrosine, and any protein with valine at position 379 was inactive. This indicated a clear necessity for an aspartate at position 379 of PheH for tPH to make DOPA. Subsequent mutations were designed to determine whether the V379D mutation alone was sufficient to allow DOPA formation, or if other substitutions were necessary. The V379D enzyme was only slightly active, indicating that additional substitutions could contribute to the ability of PheH to hydroxylate tyrosine. Mutation S251A or A322S in combination with V379D had no effect on the relative inability of V379D tPH to hydroxylate tyrosine. Mutation H264Q or Y277H in conjunction with V379D did improve the ability of the enzyme to form DOPA, and the proteins which were most active contained all three changes, H264Q, Y277H, and V379D.

Table 2: Tyrosine Hydroxylation Activities of Mutants of the Phenylalanine Hydroxylase Catalytic Domain[a]

enzyme	activity
tPH	0
S251A tPH	0
S251A/H264Q tPH	0
S251A/H264Q/Y277H tPH	0
S251A/H264Q/Y277H/A322S tPH	0
S251A/H264Q/Y277H/A322S/V379D tPH	66.2
S251A/H264Q/Y277H/A322S/V379D/Y356H tPH	27.9
S251A/H264Q/Y277H/A322S/V379D/Y356H/L293M tPH	27.6
L293M tPH	1.7
V379D tPH	6.3
A322S/V379D tPH	7.8
S251A/V379D tPH	10.1
H264Q/V379D tPH	31.2
Y277H/V379D tPH	18.2
S251A/H264Q/V379D tPH	44.0
H264Q/Y277H/V379D tPH	65.0
S251A/H264Q/Y277H/V379D tPH	68.4

[a] Conditions: 2.2 mM tyrosine, 350 μM BH_4, 50 mM HEPES/NaOH, 100 μg/mL catalase, 10 μM ferrous ammonium sulfate, and 1 mM DTT (pH 6.7 and 32 °C). The units of activity are nanomoles of DOPA produced per minute per milliliter of enzyme.

Activity of Full-Length PheH and TyrH Mutants. The mutations at the three positions that were identified by screening truncated phenylalanine hydroxylases were next incorporated into the full-length forms of both PheH and TyrH. Specifically, this included the H264Q, Y277H, and V379D PheH proteins and the Q310H, H323Y, and D425V TyrH proteins. All six single-mutation enzymes along with the multiple-mutation enzymes H264Q/V379D PheH and H264Q/Y277H/V379D PheH were purified to homogeneity. Perhaps because no substitution was made that was not modeled on the wild-type structure of a homologous enzyme, it was not difficult to obtain suitable quantities of enzymes for study.

Steady-state kinetic parameters with tyrosine and phenylalanine as the amino acid substrates for this series of mutant enzymes and the wild-type enzymes were measured.[3] As thoroughly described elsewhere (*47*−*49*), incubation of PheH with phenylalanine or with lysophosphatidylcholine results in activation of the enzyme. Although the effect is greater (10-fold) with BH_4, it is also seen with 6-MPH_4. For this reason, enzymes were pretreated with phenylalanine when that amino acid was the substrate and with lysophosphati-

[3] The concentration of oxygen was not varied in these analyses. Oxygen binds in rapid equilibrium to both TyrH and PheH (*44*−*46*) so that oxygen is saturating at all concentrations.

Table 3: Steady-State Kinetic Parameters for Amino Acid Hydroxylation by Mutant Tyrosine and Phenylalanine Hydroxylases[a]

enzyme	V_{max} for tyrosine hydroxylation (min^{-1})	K_{tyr} (μM)	V/K_{tyr} (μM^{-1} min^{-1})	V_{max} for phenylalanine hydroxylation (min^{-1})	K_{phe} (μM)	V/K_{phe} (μM^{-1} min^{-1})
TyrH	150 ± 14^b	16 ± 3^b	9.38 ± 2.0	96 ± 12	100 ± 15	0.96 ± 0.19
Q310H TyrH	128 ± 13^b	54 ± 12^b	2.37 ± 0.58	122 ± 10	8.8 ± 2	13.9 ± 3.3
H323Y TyrH	159 ± 19^b	92 ± 20^b	1.73 ± 0.43	437 ± 39	7.7 ± 1.4	56.8 ± 3.7
D425V TyrH	1.24 ± 0.9^c	45 ± 8^d	0.028 ± 0.021	270 ± 19	1.25 ± 0.2	225 ± 41
PheH	0.08 ± 0.007^c	$>10000^d$	$(0.036 \pm 0.003) \times 10^{-3}$	960 ± 41	252 ± 54	3.8 ± 0.8
H264Q PheH	0.05 ± 0.012^c	$>10000^d$	$(0.023 \pm 0.006) \times 10^{-3}$	840 ± 129	569 ± 191	1.48 ± 0.2
Y277H PheH	0.06 ± 0.036^c	$>10000^d$	$(0.027 \pm 0.0016) \times 10^{-3}$	604 ± 61	574 ± 128	1.05 ± 0.26
V379D PheH	0.05 ± 0.004^c	$>10000^d$	$(0.023 \pm 0.002) \times 10^{-3}$	38 ± 2	191 ± 27	0.2 ± 0.03
H264Q/V379D PheH	1.2 ± 0.012^c	146 ± 33^d	$(8.2 \pm 0.8) \times 10^{-3}$	281 ± 16	1080 ± 148	0.26 ± 0.04
H264Q/Y277H/V379D PheH	1.83 ± 0.077^c	1310 ± 370^d	$(1.4 \pm 0.4) \times 10^{-3}$	125 ± 15	218 ± 37	0.57 ± 0.12

[a] Conditions: varied concentrations of phenylalanine or tyrosine, 350 μM 6-MPH_4, 50 mM HEPES/NaOH, 100 μg/mL catalase, 10 μM ferrous ammonium sulfate, and 1 mM DTT (pH 6.7 and 32 °C). [b] Determined with varied concentrations of tyrosine and 350 μM 6-MPH_4, using the colorimetric assay for DOPA described in Experimental Procedures. [c] Determined with 2.2 mM tyrosine and 350 μM 6-MPH_4, using the assay for tritium release from tritiated tyrosine described in Experimental Procedures. [d] Determined with varied concentrations of tyrosine and 350 μM 6-MPH_4, using the assay for tetrahydropterin oxidation described in Experimental Procedures.

dylcholine when tyrosine was the substrate. Mutants of TyrH and nT-PheH were not activated by either treatment.

The substitution of valine for Asp425 in TyrH had a marked effect on the ability of TyrH to form DOPA (Table 3). The V_{max} value for DOPA formation by D425V TyrH decreased 120-fold, while the V/K_{tyr} value for D425V TyrH was decreased about 300-fold. Consequently, the K_{tyr} value was increased relative to that of the wild-type enzyme. The other two mutations, Q310H and H323Y, while not causing dramatic changes in the ability of TyrH to catalyze DOPA formation, did effect decreases in the V/K_{tyr} value of 4- and 5-fold, respectively, with no changes in the V_{max} values. None of the mutated tyrosine hydroxylases exhibited any change in the K_M value for 6-MPH_4 (data not shown).

All three mutated tyrosine hydroxylases were more active at phenylalanine hydroxylation than wild-type TyrH (Table 3). While there were moderate increases in V_{max} values (1.3−4.5-fold), the V/K_{phe} values showed dramatic changes, increasing 14-fold (Q310H TyrH) to 225-fold (D425V TyrH). The three enzymes also exhibited decreased K_{phe} values compared to that of wild-type TyrH. The greatest change was seen in D425V TyrH, whose K_{phe} value was 80 times lower than that of TyrH. Clearly, D425V TyrH is a very poor tyrosine hydroxylase but a quite good phenylalanine hydroxylase.

Wild-type PheH has been reported to be totally incapable of hydroxylating tyrosine to form DOPA (*43*). We found that, by using much higher than normal tyrosine (2.2 mM) and enzyme (1 μM) concentrations, we were able to measure a small amount of DOPA synthesis by PheH. The rate of DOPA formation by PheH under these conditions is 1900-fold lower than the value for TyrH (Table 3). While replacement of Asp425 of TyrH with a valine changed TyrH into a very active PheH, the reverse mutation, substituting aspartate for valine at position 379 in PheH, did not in itself significantly improve the ability of PheH to hydroxylate tyrosine (Table 3). Nor were any of the other single site-mutated PheH proteins better at DOPA synthesis than wild-type PheH. Incorporation of both the V379D and H264Q mutations did generate a PheH with significantly greater tyrosine hydroxylation activity, and the triply mutated H264Q/Y277H/V379D PheH had the highest activity (Table 3).

Because of the very low rates of tyrosine hydroxylation, it was not possible to determine V/K_{tyr} values for this series of PheH enzymes by measuring rates of DOPA formation. When tyrosine is used as a substrate for wild-type PheH, a large excess of tetrahydropterin is consumed over the amount of DOPA produced, although the enzyme does not oxidize tetrahydropterin in the absence of an amino acid (*50*). Because of this phenomenon, it proved possible to determine rates of tetrahydropterin oxidation as a function of tyrosine concentration for all of the PheH mutants. This provided a method of determining the concentration dependence of turnover by these enzymes, since the concentration dependencies will be independent of the method of assay. In the case of wild-type PheH, H264Q PheH, Y277H PheH, and V379D PheH, the initial rate of 6-MPH_4 oxidation was directly proportional to the concentration of tyrosine. This establishes that the K_{tyr} values for these enzymes are well above 10 mM. At substrate concentrations well below the K_M value, the rate of an enzyme-catalyzed reaction simplifies to the V/K value times the substrate concentration. Consequently, the V/K_{tyr} values for these four enzymes could be calculated by dividing the rate of DOPA formation determined at 2.2 mM by that concentration. The resulting values are given in Table 3. H264Q/V379D PheH and H264Q/Y277H/V379D PheH exhibited significantly greater rates of DOPA formation at 2.2 mM tyrosine than any of the single-site mutants. When the rate of tetrahydropterin oxidation was determined as a function of tyrosine concentration for these two enzymes, saturation behavior was seen, allowing K_{tyr} values to be determined. In the case of H264Q/V379D PheH, 2.2 mM is effectively a saturating tyrosine concentration, so the rate of DOPA formation determined at this concentration is a valid measure of the V_{max} value. The K_{tyr} value for H264Q/Y277H/V379D PheH is somewhat higher, so the rate of DOPA formation at 2.2 mM tyrosine underestimates the V_{max} value by about 40%. With both enzymes, the V/K_{tyr} value was calculated using these K_{tyr} and V_{max} values. Both mutant proteins exhibit large increases in their V/K_{tyr} values, although neither value approaches that seen with tyrosine hydroxylase.

The kinetic parameters for phenylalanine hydroxylation were also altered by the mutations in phenylalanine hydroxylase (Table 3). Introduction of the V379D mutation, either

alone or in combination with other mutations, had the greatest effect. The V/K_{phe} value decreased 20-fold from the wild-type value in all three enzymes containing this mutation. The V_{max} values for the mutated enzymes also decreased significantly in enzymes containing the V379D mutation. The largest decrease, 25-fold, was seen with V379D PheH.

Activity of Active Site Mutants of nT-PheH. Previous studies had shown that if the R domain of TyrH is substituted for the R domain of PheH, the substrate specificity of PheH is not altered (*35*). Still, there remained the possibility that the R domain of PheH limits alteration of substrate specificity in the C domain. Therefore, we introduced active site mutations into the chimeric protein nT-PheH, which consists of the C domain of PheH and the R domain of TyrH (*35*). The mutant enzymes V379D nT-PheH, H264Q/V379D nT-PheH, Y277H/V379D nT-PheH, and H264Q/Y277H/V379D nT-PheH were overexpressed and purified. Activities for DOPA formation by these mutated enzymes were determined at one high tyrosine concentration. The results corroborate the results obtained from the PheH constructs (data not shown). None of the chimeric mutants was a good tyrosine hydroxylase. The triple mutation gave the highest activity, about 10-fold higher than that of the simple chimera nT-PheH (3.3 vs 0.34 min^{-1}). While this was the highest activity of all the phenylalanine hydroxylases studied, it was within a factor of 2 of the levels seen with the nonchimeric enzymes. This small difference eliminates the possibility that active site mutations were prohibited from changing the substrate specificity of the C domain by the action of the R domain of PheH.

DISCUSSION

This work was undertaken to study the structural basis for the amino acid substrate specificity of the amino acid hydroxylases. The specificity is interesting from several viewpoints. From the biological point of view, the hydroxylases are crucial regulators of different branches of vertebrate metabolism, yet evolved from a single source (*10*, *16*). This source, an ancient aromatic amino acid hydroxylase, may have resembled the monomeric PheH from *Chromobacterium violaceum* (*51*, *52*). Figure 1 graphically shows that there are many points of nonidentity between rat TyrH and rat PheH. However, in the 335 amino acids which encompass the C domains of TyrH and PheH, there are only 74 residues which are not similar or identical. Some or all of these 74 differences were necessary to finely hone the substrate specificities of the two enzymes during their evolution. Phenylalanine catabolism was presumably a necessary metabolic pathway for emerging life forms before neurotransmitter synthesis. It is estimated that the gene for TyrH deviated from the ancestral hydroxylase about 750 million years ago and the gene for TrpH 600 million years ago (*10*), explaining why TyrH alone acquired the ability to hydroxylate tyrosine. From the chemical standpoint, it is intriguing that such very similar enzymes differ vastly in their ability to hydroxylate tyrosine. Both PheH and TrpH are nearly unable to perform this reaction, which is chemically easier than the hydroxylation of the less reactive aromatic rings of phenylalanine and tryptophan, reactions that they readily catalyze. Indeed, since PheH is capable of hydroxylating norleucine (*47*), an aliphatic substrate, it is particularly striking that it does not react with tyrosine.

As a first approach to analyzing the structural basis for the abilities of these two enzymes to discriminate between tyrosine and phenylalanine, active site residues which differ between the two were assayed for the ability to confer tyrosine hydroxylating activity on the catalytic domain of phenylalanine hydroxylase. This enzyme was chosen for initial screening because the discrimination by PheH against tyrosine is so profound. Indeed, the tyrosine hydroxylase activity of wild-type PheH is very close to the limits of detection. The initial series of single-site mutations identified only Val379 of PheH as critical, consistent with this mutation having the largest effect in the purified protein. In the context of the V379D mutation, two additional residues could be identified as improving the ability of PheH to hydroxylate tyrosine, His264 and Tyr277. In the purified proteins, mutations of these two residues have smaller effects than the V379D mutation, suggesting that their activities were simply below our limits of detection in the crude system. Mutagenesis of the remaining four residues either had no effect on the ability of the V379D enzyme to hydroxylate tyrosine or inhibited it. We cannot rule out the possibility that these residues make minor contributions to the amino acid specificity of PheH and TyrH, but have focused for the present on those residues for which clear changes in specificity were detected, His264, Tyr277, and Val379 in PheH and the homologous residues in TyrH, Gln310, His323, and Asp425.

The critical kinetic parameter in describing the substrate specificity of an enzyme is the V/K value for that substrate. In the case of several of the PheH enzymes, it was not possible to measure V_{max} values due to the very high K_M values of these enzymes for tyrosine. However, V/K values could still be measured, since this kinetic parameter is determined at subsaturating substrate concentrations. Figure 2 compares the substrate specificities of the wild-type and mutant enzymes. The ratio of the V/K value with phenylalanine as the substrate to the V/K value with tyrosine as the substrate is used as the measure of the relative specificities of these enzymes for the two amino acids. Qualitatively, the effects on specificity at homologous residues in the two enzymes are symmetrical. The largest effect is seen upon mutation of either Asp425 of TyrH or Val379 of PheH to the corresponding residue in the other enzyme. Decreasing effects are seen upon interconversion of His323 with Tyr277 or Gln310 with His264.

The effects of the single mutations on the specificity of TyrH are much greater than the effects on the specificity of PheH. When Asp425 of TyrH is mutated to valine, the $(V/K_{phe})/(V/K_{tyr})$ value is increased 80000-fold compared to that of wild-type TyrH. This is a remarkable effect on substrate specificity upon the substitution of a single residue. With valine at this position, TyrH is nearly incapable of DOPA synthesis; this single replacement causes a 340-fold decrease in the V/K_{tyr} value. Not only does D425V TyrH show a loss of tyrosine hydroxylation activity, it also shows a striking improvement in capacity as a phenylalanine hydroxylase. Wild-type TyrH hydroxylates phenylalanine, but with $^1/_{10}$ the V_{max} value and $^1/_4$ the V/K_{phe} value of PheH. In contrast, D425V TyrH has a 60-fold higher V/K_{phe} value than even wild-type PheH, while the V_{max} value is 30% of that of PheH.

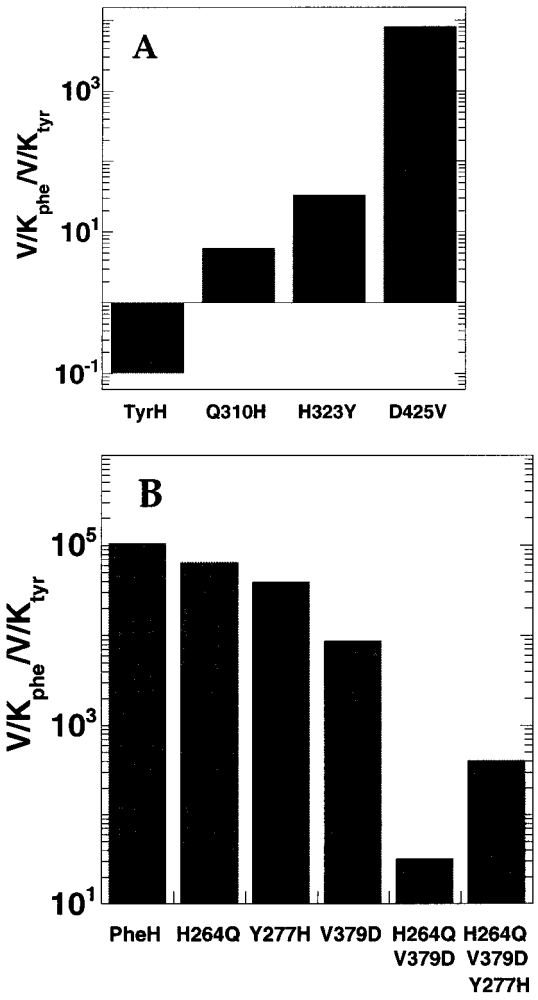

FIGURE 2: Relative amino acid substrate specificities of TyrH and PheH mutant proteins. (A) Relative specificities for phenylalanine and tyrosine of TyrH mutant proteins. (B) Relative specificities for phenylalanine and tyrosine of PheH mutant proteins. The data are from Table 3.

The change in the specificity of D425V TyrH with tyrosine as the substrate is reflected primarily in the V_{max} value for DOPA formation, while the increased specificity with phenylalanine is reflected primarily in the K_{phe} value, with much smaller changes in the V_{max} value for tyrosine formation. To the extent that the K_M values for the amino acid substrates reflect actual binding affinities, these results suggest that the discrimination against tyrosine is occurring during catalysis, while the improved ability to utilize phenylalanine as a substrate is due in part to an increased affinity for this amino acid. D425V TyrH has a K_{phe} that is 200-fold lower than that of wild-type PheH. This implies that evolution could readily have produced a PheH with a K_{phe} value much lower than that of the liver enzyme. However, such a low K_{phe} value would not be compatible with the physiological role of this enzyme, which is to metabolize excess phenylalanine. Clearly, the K_{phe} value must be sufficiently high that the cell's stores of phenylalanine for protein biosynthesis are not depleted. The regulatory properties of PheH, especially the need for activation by phenylalanine, further decrease the activity of the enzyme in the absence of the increased levels of serum phenylalanine which result after protein digestion (*29*, *53*).

Mutagenesis of His323 of TyrH to tyrosine has a smaller effect on specificity than does mutagenesis of Asp425, but the preference for phenylalanine is still increased by 3 orders of magnitude. Most of the change in specificity in this case is due to an increased V/K_{phe} value, in that the V/K_{tyr} value decreases only 5-fold. Indeed, this mutation has no effect on the V_{max} value with tyrosine. Thus, the effect is due to an improved ability to utilize phenylalanine as a substrate rather than a greatly increased level of discrimination against tyrosine.

Mutagenesis of Gln310 in TyrH to histidine has the smallest effect of the three residues identified in this study, with a 60-fold increase in the specificity for phenylalanine. As was the case with H323Y TyrH, most of the change is due to an improved ability to use phenylalanine as a substrate. The V_{max} values with either substrate are not significantly different from the values found with the wild-type enzyme, so the change in specificity is reflected entirely in the change in the K_M values for the two substrates.

The relative magnitudes of the effects of the three single mutations in PheH are in the same order as seen in TyrH, with the V379D alteration having the greatest effect and H264Q the least. However, the effects of single mutations in PheH are much less dramatic than those in TyrH. Even in the case of the V379D mutation, which has the largest effect of the three, there is only a 12-fold decrease in the preference for phenylalanine. This change in specificity is due completely to a decrease in the ability to use phenylalanine as a substrate, with no significant increase in the activity with tyrosine. It is only when multiple mutations are combined in a single PheH that the ability to use tyrosine as a substrate improves. Incorporation of both the H264Q and V379D mutations has the greatest effect, resulting in a 3000-fold change in specificity, while incorporation of all three mutations has a smaller effect. These last two enzymes clearly still prefer phenylalanine as a substrate by 2−3 orders of magnitude. Thus, it will apparently be much more difficult to modify phenylalanine hydroxylase to generate a tyrosine hydroxylase than vice versa.

Structures are available for the catalytic domains of both PheH and TyrH in the absence and presence of dihydrobiopterin (*18*, *19*, *54*, *55*), but no structures are available with amino acid substrates bound. Still, results of site-directed mutagenesis of active site residues and of modeling based on NMR data make it possible to identify the amino acid binding site in these enzymes. A representation of the active site appears in Figure 3. The active site cleft is a 17 Å deep pocket, and pterin binds at the deep end of the depression. The iron is situated approximately 10 Å into the cleft; the iron ligands in TyrH are His331, His336, and Glu376 (*19*, *56*). Ser395 hydrogen bonds to His331, holding it in the correct position for hydroxylation (*57*). The catalytic site can be seen as having a pterin side and an amino acid side, with the iron lying between them. The amino acid substrate binds at the shallow end of the pocket, and makes connections to both sides of the cleft. Mutagenesis of Arg316 and Asp328 of TyrH has shown that these residues are important in tyrosine binding, presumably by interacting with the carboxylate and amino moieties (*22*). Modeling based upon NMR analyses of PheH suggests that the aromatic rings of Phe331 and Trp326 in that enzyme (Phe377 and Trp372 in TyrH) form hydrophobic interactions with the side chain of

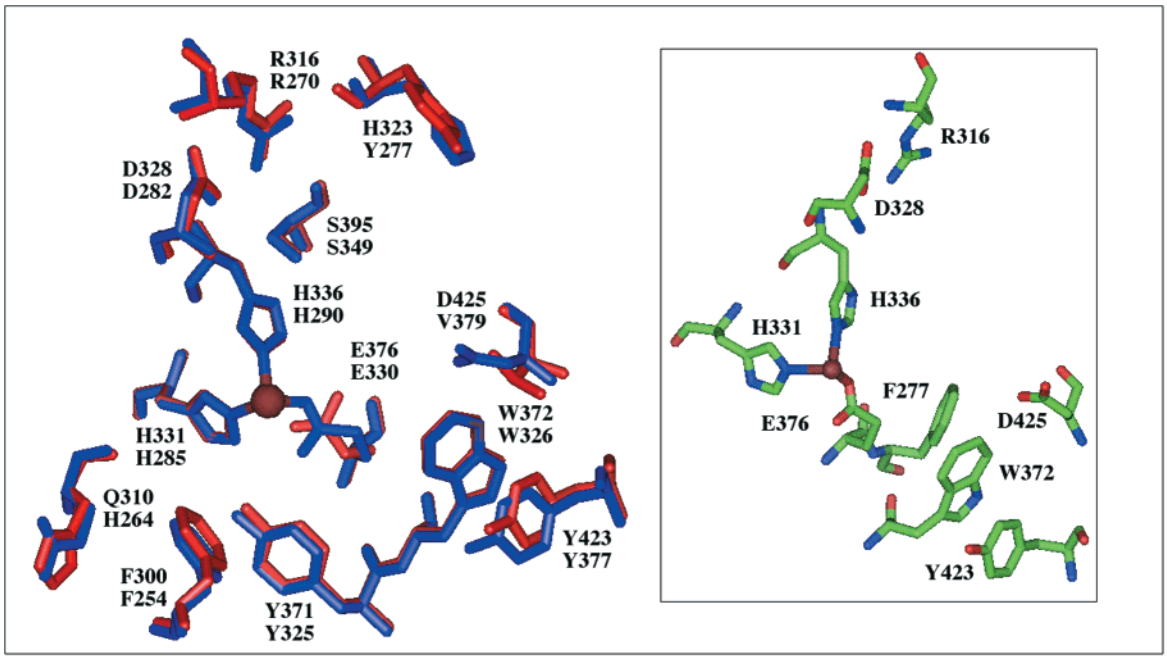

FIGURE 3: Active site topology of TyrH and PheH. Residues mentioned in the text were displayed using PDB files 1TOH and 1PAH and the visualization program InsightII. TyrH residues are in blue, and PheH residues are in red. The residues marked H331/H285, H336/H290, and E376/H330 are the ligands to the iron, shown in brown. The lower left corner of the figure is in general the area termed the pterin side of the iron, and the region to the upper right is the aromatic amino acid side of the iron. His323 and/or Tyr277 and Asp425 and/or Val379 lie toward the open end of the active site cleft, and Gln310 and/or His264 lie deep at the bottom of the cleft. The model pictured in the inset highlights the possible π-stacked sandwich between Tyr377, Trp372, and Tyr423 of TyrH.

the amino acid substrate (*58*). The aromatic rings of Trp372, Phe377, and Tyr423 (TyrH numbering) are 3.4 Å apart, the expected spacing for an aromatic π-stacked sandwich. Asp425 is positioned directly behind these three amino acid residues. These residues are all on the shallow side of the active site cleft where the amino acid substrate binds.

The tetrahydropterin binds on the other side of the cleft in both TyrH and PheH, in a π-stacking interaction with Phe300 (Phe254 in PheH) (*18*, *23*, *55*). The carboxylate of Glu286 (TyrH Glu322) and the main chain carbonyl of His264 (Gln310 in TyrH) interact with the pyrimidine ring of the pterin, either directly or through intervening water molecules (*22*, *55*, *59*). Tyr371 of TyrH interacts with the dihydroxypropyl side chain of biopterin (*55*, *58*, *60*). These residues are all on the deep side of the active site cleft where the pterin substrate binds.

In the kinetic mechanisms of both enzymes, the amino acid is the last substrate to bind (*28*, *44*, *46*). Following formation of the quaternary enzyme−substrate complex, a conformational change occurs, after which tetrahydropterin, oxygen, and iron form the hydroxylating agent, an iron oxo species (*33*, *34*, *57*, *61*, *62*). [There is no difference between the structures of the free and dihydropterin-bound forms of either enzyme (*18*, *55*).] The formation of the hydroxylating agent is both rate-limiting and the first irreversible step in the mechanism (*34*, *45*). In both enzymes, a flexible loop lies over the amino acid binding side of the cleft; in TyrH this loop contains residues 423−428 and in PheH residues 378−381. In both enzymes, this loop contains the amino acid residue identified as having the greatest effect on the substrate specificity. As water must be excluded from the hydrophobic active site for the hydroxylation reaction to occur, it is likely that this loop closes over the top of the catalytic cleft as part of the conformational change that occurs after the substrates bind. This movement could place Asp425 and/or Val379 in position to interact with the side chain of the amino acid substrate. A reasonable possibility is that a carboxyl oxygen of Asp425 is necessary to hydrogen bond to the hydroxyl group of tyrosine, to properly orient that substrate in the active site. The K_{tyr} value of D425V TyrH is only slightly greater than that of the wild-type enzyme, but hydrogen bonds in proteins are often worth less than 1 kcal/mol of binding energy. There is a much more dramatic change in the K_{phe} value, consistent with a significantly improved hydrophobic interaction due to replacement of the carboxylate with a methyl group. Alternatively, Asp425 and/or Val379 may be involved in orienting the flexible loop so that the active site has the proper shape to bind the specific amino acid substrate. The carboxylate oxygen of Asp425 is only 4 Å away from the phenolic oxygen of Tyr423. Formation of a hydrogen bond between these two residues upon loop closure could contribute to the positioning of not only Tyr423 but also Trp372 and Phe377, in that all three residues form a π-stacked aromatic sandwich as mentioned above. In such a model, Asp425 would be required to position the aromatic rings of these three residues to create an active site suited for tyrosine but not phenylalanine hydroxylation. A third possibility is that Asp425 is involved in proton transfers that accompany hydroxylation of tyrosine, but analogous proton transfers must occur in phenylalanine hydroxylation; this possibility is unlikely.

His323 is also located on the aromatic amino acid side of the catalytic cleft, but on the wall opposite Asp425, appropriately placed for the loop containing Asp425 to dock against in the closed complex. If so, replacement of tyrosine with histidine could have subtle effects on the orientation of residues in the mobile loop. The larger side chain of the tyrosine residue may constrict the amino acid site slightly,

making it more difficult to bind tyrosine. The increased hydrophobicity of the phenolic side chain of tyrosine would also favor phenylalanine over tyrosine as a substrate.

Gln310 and/or His264 lies near the bottom of the active site cleft, on the side opposite Asp425 and/or Val379. In the case of this residue, the side chain is buried, and the backbone carbonyl protrudes into the active site. The properties of Q310H TyrH are reminiscent of those of Y371F TyrH, which is also on the deep side of the cleft (*60*). The K_{phe} value of Q310H TyrH is 10-fold lower and the V/K_{phe} value 9-fold higher than those of TyrH (*60*). Both Q310H TyrH and H264Q PheH have unchanged V_{max} values, suggesting that chemical steps are not greatly affected by this mutation. The effects in this case may be due to a more constricted active site when histidine replaces the smaller glutamine.

The effects of single mutations on the specificity of PheH are much less pronounced than is the case with TyrH. When the V379D mutation is placed in PheH, the enzyme is clearly not rendered a tyrosine hydroxylase. Even the double PheH mutant H264Q/V379D still shows a strong preference for phenylalanine as a substrate. In contrast, all three TyrH single-site mutants have a preference for phenylalanine as a substrate. While wild-type TyrH has significant phenylalanine hydroxylase activity, the active site of PheH appears to be designed to prevent tyrosine hydroxylation. Not only is the rate of DOPA production by wild-type PheH very low, but the K_M value for tyrosine is extremely high, suggesting that this active site has evolved to exclude tyrosine. Comparison of the structures of PheH and TyrH shows a subtle distinction in the metal ligands. As shown in Figure 3, the glutamate ligand interacts differently with the metal in the two enzymes. In the NMR-based model of PheH with phenylalanine bound, this orientation places the $O^{\epsilon 1}$ of the glutamate where a para substituent on the substrate would be (*58*). Thus, the inability to hydroxylate tyrosine may be intrinsic to the arrangement of the metal ligands of PheH. However, it should be noted that it has been questioned whether the differences in metal ligands observed in the crystal structures are properties of the enzymes in solution (*63*).

In the study presented here, we identify for the first time a unique role for residue Asp425 of TyrH in determining the amino acid specificities of these two amino acid hydroxylases. The evolution of the ancient aromatic amino acid hydroxylase into a tyrosine hydroxylase presumably required this mutation. Mutation of valine to aspartate would have been a one-base event if the gene for the PheH of 750 million years ago contained a GTT or GTC codon. This one-base change may have been a powerful trigger for the first biosynthesis of L-DOPA.

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