Characterization of Chimeric Pterin-Dependent Hydroxylases: Contributions of the Regulatory Domains of Tyrosine and Phenylalanine Hydroxylase to Substrate Specificity†

S. Colette Daubner,‡ Patrick J. Hillas,‡ and Paul F. Fitzpatrick*,‡,§

Department of Biochemistry and Biophysics and Department of Chemistry, Texas A&M University, College Station, Texas 77843-2128

Received May 13, 1997; Revised Manuscript Received July 17, 1997⊗

ABSTRACT: Tyrosine and phenylalanine hydroxylases contain homologous catalytic domains and dissimilar regulatory domains. To determine the effects of the regulatory domains upon the substrate specificities, truncated and chimeric mutants of tyrosine and phenylalanine hydroxylase were constructed: Δ117PAH, the C-terminal 336 amino acid residues of phenylalanine hydroxylase; Δ155TYH, the C-terminal 343 amino acid residues of tyrosine hydroxylase; and 2 chimeric proteins, 1 containing the C-terminal 331 residues of phenylalanine hydroxylase and the N-terminal 168 residues of tyrosine hydroxylase, and a second containing the C-terminal 330 residues of tyrosine hydroxylase and the 122 N-terminal residues of phenylalanine hydroxylase. Steady-state kinetic parameters with tyrosine and phenylalanine as substrate and the need for pretreatment with phenylalanine for full activity were determined. The truncated proteins showed low binding specificity for either amino acid. Attachment of either regulatory domain greatly increased the specificity, but the specificity was determined by the catalytic domain in the chimeric proteins. All three proteins containing the catalytic domain of phenylalanine hydroxylase were unable to hydroxylate tyrosine. Only wild-type phenylalanine hydroxylase required pretreatment with phenylalanine for full activity with tetrahydrobiopterin as substrate.

Tyrosine hydroxylase (TYH)[1] and phenylalanine hydroxylase (PAH), along with the less-studied tryptophan hydroxylase (TRPH), are members of a group of enzymes which hydroxylate aromatic amino acids (*1*). They all utilize tetrahydrobiopterin and molecular oxygen as substrates, and each contains a single non-heme iron atom per subunit. All three are rate-limiting catalysts for important metabolic pathways: TYH, catecholamine biosynthesis; PAH, phenylalanine catabolism; and TRPH, serotonin biosynthesis. In addition to the functional similarities, these enzymes are similar in structure. They are multimers of a single subunit (2−5) which appears to consist of at least two domains. One domain (C domain) constitutes a catalytic unit and is composed of approximately 330 amino acids at the C-terminus of the subunit. The other (R domain) serves regulatory functions (*6*, *7*) and is made up of the N-terminus of the protein [about 90 residues for TRPH, 120 for PAH, and 160 for TYH (*6*, *8*, *9*)]. TYH and PAH are 75% identical in their amino acid sequences in the 330 C-terminal region, and all three proteins are 60% identical in this region (*10*). In the N-terminal domain, the three display little identity. Bacterial forms of PAH have been isolated; one from *Chromobacterium violaceum* contains 315 amino acids and is 30% identical to the C-terminal domain of rat PAH (*7*, *11*). These observations suggest that eukaryotic PAH and TYH are derived from the same ancestral gene and that the 5′ ends arose via recruitment of exons from unrelated genes (*12*); the bacterial PAH would be a form which did not acquire an N-terminal regulatory (R) domain.

Given that the eukaryotic hydroxylases are composed of one domain that is very similar and one domain that is very different, it has been proposed that the functional differences are due to the specific regulatory domain (*10*, *13*). The principal such difference between the two more heavily studied hydroxylases, the tyrosine and phenylalanine hydroxylases, is their substrate specificities. Tyrosine hydroxylase is able to hydroxylate phenylalanine, but this is an inefficient process, in that more tetrahydropterin is consumed than tyrosine is produced (*14*, *15*). In contrast, phenylalanine hydroxylase is reported to be unable to hydroxylate tyrosine (*2*). Another phenomenon which distinguishes PAH from TYH is substrate activation. Phenylalanine hydroxylase is activated by incubation with phenylalanine; without such treatment, initial rates of tyrosine formation show pronounced lags so that plots of activity versus phenylalanine concentration are sigmoidal rather than hyperbolic (*16*, *17*). Tyrosine hydroxylase does not require activation by tyrosine (*18*).

We describe in this paper the construction of chimeric mutants of TYH and PAH in which the R domain of each enzyme is attached to the C domain of the other. Using

† This research was supported in part by NIH Grant GM 47291 and Robert A. Welch Foundation Grant A-1245 to P.F.F. and American Heart Association (Texas Affiliate) Grant 92R-033 to S.C.D. P.J.H. was supported by NIH Grant T32 GM 08523.

* Address correspondence to this author at the Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128. Phone: 409-845-5487. Fax: 409-845-9274. E-mail: fitzpat@bioch.tamu.edu.

‡ Department of Biochemistry and Biophysics.

§ Department of Chemistry.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1997.

[1] Abbreviations: DOPA, dihydroxyphenylalanine; PEI, polyethylenimine; TYH, tyrosine hydroxylase; PAH, phenylalanine hydroxylase; nP-TYH, the chimeric protein containing the N-terminal domain of PAH and the C-terminal domain of TYH; nT-PAH, the chimeric protein containing the N-terminal domain of TYH and the C-terminal domain of PAH; MePH4, 6-methyltetrahydropterin; DHPR, sheep dihydropterin reductase; K_{phe}, K_M value for phenylalanine; K_{tyr}, K_M value for tyrosine.

S0006-2960(97)01113-6 CCC: $14.00

Table 1: Oligonucleotides Used To Introduce or Remove Restriction Sites

enzyme	oligonucleotide designation	oligonucleotide sequence	effect of mutagenesis
PAH	PHNEWNCO	5′-ctgag agcca gcatg gccat ggcag ctgtt gtc-3′	introduced *Nco*I site at start codon of cDNA
PAH	PHΔNCO	5′-tacaa ctacc gccac ggcca gccca tccct-3′	removed existing *Nco*I site at position 510[a]
PAH	PHNEWSAC	5′-gtgcc ctggt tcccg cggac cattc agg-3′	introduced *Sac*II site at position 367
PAH	PHNCO6	5′-aagga aaaga acacc atggc ctggt tccca aggac c-3′	introduced *Nco*I site at position 349
TYH	THNEWNCO	5′-cacca gcttg cccca tggcc acccc cagcg-3′	introduced *Nco*I site at start codon of cDNA
TYH	THNEWSAC	5′-gtccc ctggt tcccg cggaa agtgt cgg-3′	introduced *Sac*II site at position 505

[a] Positions are relative to A of start codon of native gene.

these chimeric mutants, as well as truncated mutants lacking their N-terminal R domains, and the wild-type enzymes, we have studies the roles of the amino-terminal domains in defining the amino acid substrate specificity of these enzymes. We have focused on three properties: (1) the effects of the N-termini of TYH and PAH on K_M values for amino acid substrates and on V_{max} values for catalysis; (2) whether the N-terminus of PAH alone is sufficient for substrate activation, and (3) the effect of the N-termini on the extent of the coupling of amino acid hydroxylation and tetrahydropterin oxidation.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were custom-synthesized on an Applied Biosystems Model 380B DNA synthesizer by the Gene Technology Laboratory of the Biology Department of Texas A&M University. Tritiated tyrosine was from Amersham. 6-Methyltetrahydropterin was synthesized as described previously (*19*). (6*R*)-Tetrahydrobiopterin was purchased from Calbiochem. Plasmid pTZ18R and helper phage M13KO7 were from Pharmacia. Plasmid pET23d was from Novagen. Restriction and DNA modification enzymes were purchased from New England Biolabs and Promega. Plasmids were purified using the Wizard mini-prep kit of Promega. Catalase was obtained from Boehringer Mannheim. Agarose (SeaKem) was from FMC. Chromatography resins were as follows: Procion Red (Amicon Red A), Amicon; phenyl-Sepharose Fast Flow, Q-Sepharose Fast Flow, and Sephacryl S-300, Pharmacia; ceramic hydroxyapatite, BioRad. *E. coli* strains BL21(DE3) and BL21(DE3)-pLysS were obtained from Novagen; these strains were used for protein expression. *E. coli* strain XL1-Blue was obtained from Stratagene and was used during DNA subcloning protocols. *E. coli* strain CJ236 was obtained from Invitrogen and was used for the production of single-stranded uridine-containing DNA. DNA sequencing was done by the Gene Technology Laboratory of the Biology Department of Texas A&M University.

Construction of Vectors for Enzyme Expression. All site-directed mutagenesis was carried out according to the protocol of Kunkel et al. (*20*). The sequences of the oligonucleotides used for mutagenesis appear in Table 1. Isolation of DNA fragments from agarose gels was carried out using QIA-Quick kit components from Qiagen. Ligation of DNA fragments was carried out with Promega T4 DNA ligase at room temperature for 1 h.

Plasmid pTH6, which contains the cDNA for rat TYH inserted into the *Bam*HI site of pTZ18R, with a unique *Nde*I site at the position coding for methionine 1, has been previously described (*21*). The *Nde*I site was changed to a *Nco*I site by site-directed mutagenesis, giving plasmid pTH11. A unique *Sac*II recognition site was introduced at position 505 in pTH11; the new plasmid (pTH16) was detected by *Sac*II digestion and gel electrophoresis. Plasmids pTH16 and pET23d were digested with *Nco*I and *Bam*HI; pET23d and the fragment from pTH16 which codes for TYH were purified and ligated. Correct plasmids were identified by agarose gel separation of fragments after restriction enzyme digestion with *Bam*HI alone, *Nco*I alone, *Bam*HI and *Nco*I together, *Eco*RI alone, and *Pst*I alone. The correct plasmid was named pETYH8.

A vector containing the cDNA for rat PAH was a generous gift from Dr. Ian Jennings. The cDNA was removed from this vector and inserted into the *Eco*RI site of pTZ18R by restriction enzyme digestion, fragment isolation, and ligation, giving plasmid ptzRPH1. The cDNA for PAH contained one internal *Nco*I site which was removed by site-directed mutagenesis, altering one base in the site, but leaving the amino acid sequence unchanged. This plasmid was called ptzRPH2. A unique *Nco*I site was introduced at the 5′ end of the cDNA. Detection of plasmids with mutations was accomplished by looking for the correct *Bam*HI and *Nco*I restriction fragments on agarose gels. A unique *Sac*II site was introduced at position 367, giving plasmid ptzRPH4. Plasmids ptzRPH4 and pET23d were digested with *Nco*I and *Bam*HI; pET23d and the fragment from ptzRPH4 which codes for PAH were combined as described above. Correct recombinants were identified by restriction enzyme analysis with *Bam*HI alone, *Nco*I alone, *Bam*HI and *Nco*I together, *Eco*RI alone, and *Pst*I alone. The correct plasmid was named pERPH5.

Plasmids pERPH5, pETYH8, ptzRPH4, and pTH16 were digested with *Sac*II and *Bam*HI. The digested DNA was separated on an agarose gel, and four fragments were isolated as described above. The fragment coding for the C-terminus of TYH (from pTH16) and the fragment containing pET23d plus the 5′ end of the cDNA for PAH (from pERPH5) were combined. Likewise, the fragment coding for the C-terminus of PAH (from ptzRPH4) and the fragment containing pET23d plus the 5′ end of the TYH cDNA (from pETYH8) were combined. Ligation was carried out as before. Plasmids were detected by restriction digestion and agarose gel electrophoresis, using *Bam*HI, *Nco*I, *Eco*RI, *Hin*dIII, and *Pst*I. Correct recombinants were given the following names: vector with the 5′ end of the TYH cDNA and the 3′ end of the PAH cDNA, pPCTN2; vector with the 5′ end of the PAH cDNA and the 3′ end of the TYH cDNA, pTCPN2.

Plasmid pETOHΔ155, which codes for a truncated form of TYH lacking its 155 amino-terminal residues, has already been described (*9*). To obtain a truncated form of PAH lacking its N-terminus, further mutagenesis was performed on plasmid ptzRPH2. A *Nco*I site was introduced into the sequence coding for residues 117 and 118, causing a start codon to substitute for valine 118. This plasmid was called

ptzRPH5. Plasmids ptzRPH5 and pET23d were digested with *Nco*I and *Bam*HI; the 1618 bp fragment containing the 3′ end of the PAH cDNA and the cut pET23d were isolated, combined, and ligated. The proper plasmid was detected by electrophoretic separation of restriction fragments from *Nco*I, *Bam*HI, *Bgl*II, *Hin*dIII, and *Pst*I digestion. It was called pERPHΔ117.

The coding regions of all expression plasmids were sequenced to check for unexpected mutations. All expression plasmids were introduced into competent *E. coli* of the strain BL21(DE3) already containing the lysozyme-encoding pACYC-based plasmid pLysS.

Bacterial Cell Growth. Bacterial growth was carried out at 37 °C for pERPHΔ117, pPCTN2, pETYH8, and pETOHΔ155 and at 30 °C for pERPH5 and pTCPN2. Bacteria were grown in LB medium containing either 100 μg/mL ampicillin and 50 μg/mL chloramphenicol (LB-amp-chlor) or 100 μg/mL carbenicillin and 50 μg/mL chloramphenicol (LB-carb-chlor). One isolated colony of BL21-(DE3) containing the plasmid of interest was picked from LB-agar plates (plates contained 100 μg/mL carbenicillin and 50 μg/mL chloramphenicol) no longer than 1 week after transformation. One colony was used to inoculate 50 mL of LB-carb-chlor, and the culture was incubated overnight. The overnight cultures were used to inoculate 1 L cultures of LB-amp-chlor at a ratio of 10 mL/L for 37 °C growth and 17.5 mL for 30 °C growth. Two liters of culture were grown for each enzyme preparation. When the A_{600} value of a culture reached 0.8, isopropyl β-D-thioglucanopyranoside was added to a final concentration of 0.5 mM. For growth at 37 °C, incubation continued for 3 h; for 30 °C, 5 h. Cells were harvested by centrifugation at 9000*g* for 30 min and stored at −20 °C overnight.

Protein Purification. All protein isolation steps were performed at 4 °C, with the exception of the 25 °C incubation step in the purification of wild-type PAH (*2*). Samples at every step were analyzed by activity assay and denaturing polyacrylamide gel electrophoresis. Protein concentrations were determined using the BioRad protein assay kit until after the first chromatography step of each preparation, following which absorbance at 278 nm was used. Extinction coefficients were calculated from the amino acid contents of the individual proteins using the method of Pace et al. (*22*).

(A) Phenylalanine Hydroxylase. Phenylalanine hydroxylase was purified according to the protocol of Shiman et al. (*2*). This protocol consists of cell lysis followed by chromatography on phenyl-Sepharose. The final specific activity was 7.2 μmol of Tyr/(min·mg).

(B) Tyrosine Hydroxylase. Tyrosine hydroxylase was purified as previously described (*21*). The purification protocol consists of cell lysis, ammonium sulfate precipitation (30−42% saturation), and heparin−Sepharose chromatography). The final specific activity was 2.7 μmol of DOPA/(min·mg).

(C) nT-PAH. nT-PAH was purified using the same protocol as wild-type TYH, except that the ammonium sulfate fractionation step isolated proteins precipitating between 30 and 45% saturation. The final specific activity was 4.9 μmol of Tyr/(min·mg).

(D) nP-TYH. The cell pellet was suspended in an 8-fold excess (with respect to the initial weight of cells) of 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 100 μg/mL phenylmethanesulfonyl fluoride, pH 7.1. The suspension was passed through an 18 gauge needle 3 times and sonicated with 6 bursts of 30 s at 45 W with 3 min intervals. The resulting solution was centrifuged at 27000*g* for 30 min. The resulting supernatant was brought to 0.008% polyethylenimine (PEI) by the addition of a 0.5% stock in 20 mM Tris-HCl, pH 7.0, stirred for 20 min, and centrifuged at 27000*g* for 30 min. The PEI supernatant was treated with ammonium sulfate; proteins precipitating between 32 and 48% saturation were collected and dissolved in an 8-fold volume (with respect to the initial weight of cells) of 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.1. The sample was applied to a Q-Sepharose Fast Flow column (1.5 cm × 10 cm) equilibrated with 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.1. After loading, the resin was washed with the same buffer until the A_{278} value of the filtrate was 0.03 or less. The enzyme was eluted with a gradient from 0 to 1 M NaCl in 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.1, with a total volume of 320 mL. The enzyme eluted at approximately 0.40 M NaCl. Fractions displaying approximately equal specific activities across the peak were pooled.

The Q-Sepharose pool was frozen at −70 °C in five aliquots of equal volume. One aliquot at a time was subjected to further purification no more than 2 weeks prior to use in kinetic analyses. An aliquot was diluted 3-fold with 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 15% glycerol, pH 7.1, and was applied to a 3 mL column of Procion-Red-linked agarose (Amicon Red). The column was washed after loading with 15 mL of 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 15% glycerol, pH 7.1, and then with 40 mL of 0.15 M NaCl, 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 15% glycerol, pH 7.1. The enzyme was eluted with 1.3 M NaCl, 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 15% glycerol, pH 7.1. The final specific activity was 0.82 μmol of DOPA/(min·mg).

(E) Δ155TYH. Lysis, sonication, and PEI precipitation were carried out as described above for nP-TYH. The proteins precipitating between 30 and 50% ammonium sulfate saturation were dissolved in an 8-fold volume (with respect to the cell pellet weight) of 50 mM Tris-HCl, 75 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.0, and applied to a Q-Sepharose Fast Flow column (1.5 cm × 15 cm). After loading, the resin was washed with the same buffer until the A_{278} value of the effluent was below 0.03. The enzyme was eluted with a gradient from 0 to 1 M NaCl in 50 mM Tris-HCl, 75 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 15% glycerol, pH 7.0, with a total volume of 240 mL. The enzyme eluted at approximately 0.38 M NaCl. Fractions displaying approximately equal specific activities across the peak were pooled.

The Q-Sepharose pool was concentrated in an Amicon ultrafiltration cell using a PM30 membrane to approximately 10 mg/mL protein. It was loaded onto a column of Sephacryl S-300 (2.5 cm × 35 cm) equilibrated with 50 mM Tris-HCl, 75 μM EDTA, 150 mM NaCl, and 10% glycerol, pH 7.0, and eluted with the same buffer. Fractions displaying approximately equal specific activities across the peak were

pooled, and leupeptin and pepstatin were added to a final concentration of 1 μM each. The final specific activity was 2.7 μmol of DOPA/(min·mg).

(F) Δ117PAH. Lysis, sonication, and PEI precipitation were carried out as described for nP-TYH. Ammonium sulfate fractionation isolated proteins precipitating between 30 and 55% saturation. The ammonium sulfate pellet was dissolved in an 8-fold volume (with respect to the initial weight of cells) of 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.1, and applied to a Q-Sepharose Fast Flow column (1.5 cm × 15 cm). The enzyme did not bind to Q-Sepharose under these conditions. The filtrate fractions which contained PAH activity were pooled and concentrated by precipitation at 55% ammonium sulfate saturation. The precipitate was dissolved in an 8-fold volume (with respect to the cell pellet weight) of 75 mM sodium phosphate, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.1, and applied to a ceramic hydroxyapatite column (1.5 cm × 10 cm). After loading, the resin was washed with the same buffer until the A_{278} value of the effluent was less than 0.1. The enzyme was eluted with a gradient from 50 to 350 mM sodium phosphate in 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.1, with a total volume of 500 mL. The enzyme eluted at approximately 160 mM sodium phosphate. Fractions displaying approximately equal specific activities across the peak were pooled and concentrated by ammonium sulfate precipitation. The pellet was dissolved in 50 mM Tris-HCl, 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol, pH 7.1. The final specific activity was 13.0 μmol of Tyr/(min·mg).

Assays. Two assays for the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA) were used. The release of tritium from 3,5-[^{3}H]tyrosine, as previously described (*23*), in 50 mM HEPES−tetraethylammonium hydroxide, pH 7.0, at 30 °C, was used for standard assays. The second assay was a colorimetric assay which measured directly the amount of DOPA formed (*24*). This was carried out in 80 mM HEPES−NaOH, 60 μg/mL catalase, pH 7.1, at 25 °C.

Tyrosine formation from phenylalanine was measured by monitoring absorbance changes at 275 nm due to the production of tyrosine (*17*). The assays contained 5−800 μM phenylalanine and 10−400 μM 6-methyltetrahydropterin in 80 mM HEPES−NaOH, 5 mM DTT, and 60 μg/mL catalase, pH 7.1, at 25 °C.

Rates of tetrahydropterin oxidation were determined using a coupled assay with dihydropterin reductase, monitoring the decrease in absorbance at 340 nm due to NADH oxidation (*25*). The assays contained 5−800 μM phenylalanine or 1−300 μM tyrosine in addition to 125 μM tetrahydrobiopterin or 150 μM 6-methyltetrahydropterin, 80 mM HEPES−NaOH, 60 μg/mL catalase, 200 μM NADH, and 0.05 unit/mL sheep dihydropterin reductase, pH 7.1, at 25 °C. Some assays were repeated with 3 times as much dihydropterin reductase to ensure that it was present in excess. Steady-state kinetic data were fit directly to eq 1 using the program KinetAsyst (IntelliKinetics, State College, PA).

$$v = VS/(K_M + S) \quad (1)$$

When measuring activation by phenylalanine, enzymes (6.7−17.2 μM) were preincubated with 1 mM phenylalanine or without phenylalanine at 25 °C for 15 min just prior to assay; such assays contained tetrahydrobiopterin or 6-methyltetrahydropterin as indicated in Table 3. Assay conditions were 80 mM HEPES−NaOH, 60 μg/mL catalase, 200 μM NADH, 600 μM phenylalanine, and 0.05 unit/mL sheep dihydropterin reductase, pH 7.1, at 25 °C.

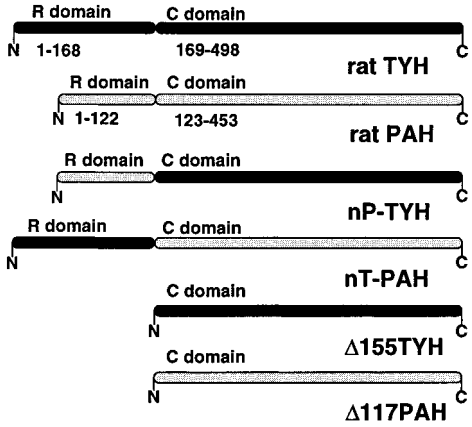

FIGURE 1: Schematic representation of the aromatic amino acid hydroxylases in the study. Each protein is identified by a name below and to the right. Regulatory (R) domains and catalytic (C) domains are indicated. Amino termini are indicated with the single letter N and carboxy termini with the single letter C. Dark-shaded segments represent polypeptide seqences from rat tyrosine hydroxylase, and lighter segments sequences from rat phenylalanine hydroxylase. The numbers underneath the R domains of the wild-type enzymes define the exact portions of the proteins by residue position which were replaced by the R domain of the other wild-type hydroxylase to form the chimeric proteins.

Scheme 1: Alignment of Amino Acid Sequences of Rat Tyrosine Hydroxylase and Rat Phenylalanine Hydroxylase[a]

TYH	M	A	T	P	S	A	P	S	P	Q	P	K	G	F	R	R	A	V	S	E	20
TYH	Q	D	A	K	Q	A	E	A	V	T	S	P	R	F	I	G	R	R	Q	S	40
TYH	L	I	E	D	A	R	K	E	R	E	A	A	A	A	A	A	A	A	A	V	60
PAH							M	A	A	V	V	L	E	N	G	V	L	S	R	K	14
TYH	A	S	S	E	P	G	N	P	L	E	A	V	V	F	E	E	R	D	G	N	80
PAH	L	S	D	F	G	Q	E	T	S	Y	I	E	D	N	S	N	Q	N	G	A	34
TYH	A	V	L	N	L	L	F	S	L	R	G	T	K	P	S	S	L	S	R	A	100
PAH	I	S	L	I	F	S	L	K	E	E	V	G	A	L	A	K	V	L	R	L	54
TYH	V	K	V	F	E	T	F	E	A	K	I	H	H	L	E	T	R	P	A	Q	120
PAH	F	E	E	N	D	I	N	L	T	H	I	E	S	R	P	S	R	L	N	K	74
TYH	R	P	L	A	G	S	P	H	L	E	Y	F	V	R	F	E	V	P	S	G	140
PAH	D	E	Y	E	F	F	T	Y	L	D	K	R	T	K	P	V	L	G	S	I	94
TYH	D	L	A	A	L	L	S	S	V	R	R	V	S	D	D	V	R	S	A	R	160
PAH	I	K	S	L	R	N	D	I	G	A	T	V	H	E	L	S	R	D	K	E	114
TYH	E	D	K	V	P	W	F	P	R	K	V	S	E	L	D	K	C	H	H	L	180
PAH	K	N	T	V	P	W	F	P	R	T	I	Q	E	L	D	R	F	A	N	Q	134
TYH	V	T	K	F	D	P	D	L	D	L	D	H	P	G	F	S	D	Q	V	Y	200
PAH	I	L	S	Y	G	A	E	L	D	A	D	H	P	G	F	K	D	P	V	Y	154
TYH	R	Q	R	R	K	L	I	A	E	I	A	F	Q	Y	K	H	G	E	P	I	220
PAH	R	A	R	R	K	Q	F	A	D	I	A	Y	N	Y	R	H	G	Q	P	I	174

[a] The residues in bold indicate identity between the two sequences; the sequence VPWFPR is where the R and C domains were switched to produce the chimeric enzymes nT-PAH and nP-TYH. Relative identity (75% over the 330 C-terminal residues) continues to within 20 residues of the carboxy termini of the proteins. Sequences have not been recorded in full here in order to save space.

RESULTS

Overexpression and Purification of Mutant Proteins. The proteins utilized in this study are portrayed in Figure 1. Based upon the amino acid sequences of tyrosine and phenylalanine hydroxylase (Scheme 1) and several studies of deletion mutants (*9*, *26*−*28*), the conserved sequence VPWFPR was taken as the beginning of the catalytic domains of these proteins. This valine is amino acid residue 164 of rat tyrosine hydroxylase and residue 118 of rat phenylalanine hydroxylase. The chimeric protein nP-TYH contains the N-terminal

Table 2: Summaries of Protein Purifications

enzyme	wet weight of cell pellet from 2 L of medium (g)	protein (mg)	specific activity [μmol/(min·mg)]
TYH	13	12	2.7
PAH	11	9	7.2
nP-TYH	38	9	0.82
nT-PAH	8	13	4.9
Δ155TYH	16	60	2.7
Δ117PAH	8	33	13

122 amino acid residues of PAH fused to the C-terminal 330 residues of TYH. The protein nT-PAH similarly contains the N-terminal 168 residues of TYH fused to the C-terminal 331 residues of PAH. In addition, Δ117PAH, a phenylalanine hydroxylase which lacks the N-terminal 117 amino acid residues, and wild-type PAH were expressed and purified. We have previously described expression and characterization of the wild-type TYH and of Δ155TYH, a mutant protein lacking the N-terminal 155 amino acid residues of TYH (*9*, *21*).

Table 2 summarizes the final mass of protein and activities obtained for each enzyme studied. The chimeric proteins, Δ117PAH, and wild-type PAH were all readily overexpressed using the pET system in strain BL21(DE3). It was noticed that loss of large quantities of protein to insoluble inclusion bodies hampered the production of proteins bearing the R domain of PAH. Bacteria producing the two such enzymes, wild-type PAH and nP-TYH, were grown at a lower temperature to decrease the problem, although losses still occurred. These two proteins were also the least stable of the six and suffered losses of activity with each freeze−thaw cycle. In contrast, the N-terminus of TYH appeared to render foreign proteins especially easy to overproduce with the pET system. Tyrosine hydroxylase and nT-PAH constituted approximately 35% of the total protein after a single ammonium sulfate fractionation. These two enzymes were also most stable upon storage and repeated freeze−thawing.

We have previously shown that the N-terminal domain of TYH binds heparin (*9*, *28*). Consistent with this, both proteins containing the R domain of TYH, wild-type TYH and nT-PAH, could be purified with a single heparin−Sepharose column. Alternative purification procedures were developed for the previously undescribed proteins nP-TYH and Δ117PAH. Although both Δ155TYH and Δ117PAH contain only the homologous portions of the two hydroxylases, neither could be purified by the technique which worked well for the other; this provided an early suggestion that the catalytic domains of TYH and PAH are distinctly different proteins.

All six enzymes were judged to be 90−99% pure by denaturing polyacrylamide gel electrophoresis. Figure 2 shows a photograph of a gel containing all six proteins.

Activation by Phenylalanine. Wild-type phenylalanine hydroxylase exhibits nonlinear rates and sigmoidal kinetics unless the enzyme is first activated with phenylalanine (*29*). The sensitivity of each enzyme to activation by phenylalanine was determined. Table 3 contains the extent of activation for all six proteins after incubation at 25 °C in the presence of 1 mM phenylalanine over incubation in the absence of phenylalanine. Assays were performed with either tetrahydrobiopterin or 6-methyltetrahydropterin. Only wild-type PAH was significantly activated by preincubation with

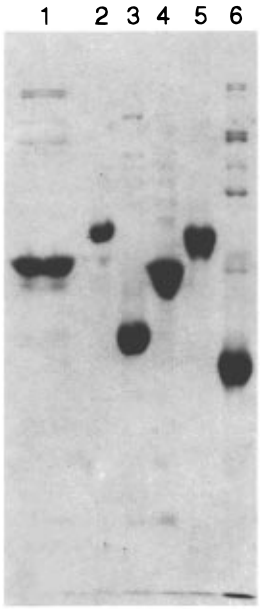

FIGURE 2: Photograph of a denaturing 12% polyacrylamide gel containing all six proteins of this study. The proteins are: lane 1, nP-TYH; lane 2, TYH; lane 3, Δ155TYH; lane 4, PAH; lane 5, nT-PAH; and lane 6, Δ117PAH. Each lane contained approximately 2−5 μg of enzyme. The bromophenol blue front is visible at the bottom of the gel; the top of the gel is included in the photograph.

Table 3: Activation of Mutant Hydroxylases by Phenylalanine[a]

enzyme	activation with $MePH_4$ as substrate[b]	activation with tetrahydrobiopterin as substrate
TYH	0.9	0.9
PAH	1.2	11.0
nP-TYH	1.1	1.0
nT-PAH	0.9	1.0
Δ155TYH	1.2	0.6
Δ117PAH	0.9	0.4

[a] Enzymes were incubated for 15 min at 25 °C in the presence of 1 mM phenylalanine or buffer lacking phenylalanine before measuring the rate of tetrahydropterin oxidation. Shown is the ratio of the rate of enzyme incubated with phenylalanine to the rate of enzyme incubated in buffer. [b] The concentrations of tetrahydropterin used were 2−3 times greater than the K_M values.

phenylalanine (11-fold). As has been previously noted (*5*), the effect was only significant with tetrahydrobiopterin as substrate; no significant effect of preincubation with phenylalanine was seen when 6-methyltetrahydropterin was used. Moreover, wild-type PAH which had not been preincubated with phenylalanine had the same V_{max} value with 6-methyltetrahydropterin as substrate as the preincubated enzyme had with tetrahydrobiopterin. Thus, only in the presence of the physiological pterin cofactor is the preincubation by substrate an important activating mechanism.

Substrate Specificities. Steady-state kinetic parameters were determined with tyrosine and phenylalanine as substrates for each of the six proteins. 6-Methyltetrahydropterin was used as the pterin substrate to avoid the necessity for pretreatment with phenylalanine. The Michaelis constant for 6-methyltetrahydropterin did not vary significantly among the six proteins; it was in the 10−30 μM range in all cases (data not shown). The concentration of the pterin was kept at least 8-fold higher than its K_M value when the concentrations of amino acids were varied. With both amino acid substrates, initial rates were determined in two ways. With phenylalanine as substrate, the rate of tyrosine forma-

Table 4: Steady-State Kinetic Parameters of Mutant Hydroxylases with Phenylalanine as Substrate

enzyme	K_{phe} (μM)	V_{max} for $MePH_4$ oxidation[b] (min^{-1})	V/K_{phe} for $MePH_4$ oxidation[b] (μM^{-1} min^{-1})	V_{max} for Tyr formation[a] (min^{-1})	coupling[c] (Tyr formed/ $MePH_4$ oxidized)
TYH	91 ± 25^a 109 ± 20^b	349 ± 31	3.2	96 ± 12	0.3 ± 0.05
PAH	309 ± 32^a 288 ± 59^b	281 ± 29	0.98	373 ± 21	1.3 ± 0.15
nP-TYH	76 ± 15^a 52 ± 17^b	113 ± 2	2.2	56 ± 5	0.5 ± 0.04
nT-PAH	98 ± 22^a 73 ± 15^b	240 ± 4	3.3	273 ± 27	1.1 ± 0.11
Δ155TYH	15 ± 6^a 6.8 ± 1^b	158 ± 11	23	80 ± 7	0.5 ± 0.06
Δ117PAH	145 ± 28^a 182 ± 35^b	409 ± 48	2.2	502 ± 57	1.2 ± 0.2

[a] Rate of tyrosine formation, determined with 80 mM HEPES, 5 mM dithiothreitol, 8−800 μM phenylalanine, 60 μg/mL catalase, and 150 μM 6-methyltetrahydropterin, pH 7.1, 25 °C. [b] Determined from rate of 6-methyltetrahydropterin oxidation in 80 mM HEPES, 8−800 μM phenylalanine, 60 μg/mL catalase, 150 μM 6-methyltetrahydropterin, 200 μM NADH, and 50 milliunits of DHPR, pH 7.1, 25 °C. [c] V_{max} from tyrosine formation/ V_{max} for tetrahydropterin oxidation.

Table 5: Steady-State Kinetic Parameters of Mutant Hydroxylases with Tyrosine as Substrate

enzyme	K_{tyr}[a] (μM)	V_{max} for $MePH_4$ oxidation[a] (min^{-1})	V/K_{tyr} for $MePH_4$ oxidation[a] (μM^{-1} min^{-1})	V_{max} for DOPA formation[b] (min^{-1})	coupling[c] (DOPA formed/ $MePH_4$ oxidized)
TYH	16 ± 3	165 ± 11	10.3	150 ± 14	0.91 ± 0.10
PAH	<100	15 ± 1	>0.15	nd[d]	0
nP-TYH	4.3 ± 1	64 ± 1	14.9	44 ± 5.2	0.69 ± 0.08
nT-PAH	82 ± 8	23 ± 0.1	0.28	nd	0
Δ155TYH	7.0 ± 1	89 ± 1	12.7	104 ± 9	1.2 ± 0.10
Δ117PAH	42 ± 6	38 ± 2	0.90	nd	0

[a] Determined from the rate of 6-methyltetrahydropterin oxidation with 80 mM HEPES, 5−300 μM tyrosine, 60 μg/mL catalase, 150 μM 6-methyltetrahydropterin, 200 μM NADH, and 50 milliunits of DHPR, pH 7.1, 25 °C. [b] Determined from the rate of DOPA formation in 80 mM HEPES, 5−300 μM tyrosine, 60 μg/mL catalase, and 150 μM 6-methyltetrahydropterin, pH 7.1, 25 °C. [c] V_{max} from tyrosine formation/V_{max} for tetrahydropterin oxidation. [d] Not detectable.

tion was determined directly, following the increase in absorbance at 275 nm. In addition, the rate of 6-methyltetrahydropterin oxidation was measured using a coupled assay with dihydropterin reductase. With tyrosine as substrate, the rate of DOPA formation was determined directly, and the coupled assay was used to measure 6-methyltetrahydropterin oxidation.

The results obtained with phenylalanine as substrate are given in Table 4. The K_{phe} values were highest for PAH and Δ117PAH and lowest for Δ155TYH. In contrast to the K_M values, the V_{max} values obtained depended upon whether rates of tyrosine formation or of 6-methyltetrahydropterin oxidation were measured. The V_{max} values for tyrosine formation were highest for the proteins with the C domain of PAH. With these three proteins, the results obtained with the two assays were indistinguishable. This was not the case for the three proteins containing the C domain of TYH. For these proteins, the rates of 6-methyltetrahydropterin oxidation were severalfold greater than the rates of tyrosine formation. Indeed, the V_{max} values for 6-methyltetrahydropterin oxidation differed by less than 4-fold among all six proteins. The V_{max} values for 6-methyltetrahydropterin oxidation in the presence of phenylalanine were highest for TYH and the proteins with the C domain of PAH; nP-TYH and Δ155TYH had lower values. The ratio of the V_{max} values for the two assays can be used to calculate the stoichiometry of 6-methyltetrahydropterin oxidation and phenylalanine hydroxylation for the individual proteins. The values for the coupling of the two reactions are given in Table 4. The three proteins which contain the C domain of PAH show stoichiometries of 1, while the three proteins which contain the C domain of TYH show significant uncoupling of 6-methyltetrahydropterin oxidation and tyrosine formation.

Steady-state kinetic parameters were also determined with tyrosine as substrate; the results are summarized in Table 5. The K_{tyr} value was measured by monitoring 6-methyltetrahydropterin oxidation. The value for PAH could not be measured due to extremely low activity; since the initial rates with this enzyme were constant above a tyrosine concentration of 200 μM, an upper limit of 100 μM can be placed on this value. The other proteins showed K_{tyr} values from 4 to 80 μM. The V_{max} values for 6-methyltetrahydropterin oxidation in the presence of tyrosine were highest for the three enzymes containing the C domain of TYH. In contrast, only the enzymes with the C domain of TYH were able to hydroxylate tyrosine to form DOPA. For these enzymes, the V_{max} values obtained with the two assays agreed reasonably well. The V_{max} values for 6-methyltetrahydropterin oxidation and tyrosine hydroxylation were used to calculate the coupling of the two partial reactions for each protein (Table 5).

DISCUSSION

In order to probe the contributions of the regulatory domains of tyrosine and phenylalanine hydroxylase to substrate specificities, we have constructed one protein containing the R domain of TYH and the C domain of PAH and another containing the C domain of TYH and the R domain of PAH (Figure 1). We have compared these enzymes with the wild-type enzymes and with truncated proteins which lack the R domains completely. In characterizing the effects of the R domains on the substrate specifici-

Table 6: Substrate Specificities of Mutant Hydroxylases[a]

enzyme	K_{phe}/K_{tyr}	$V_{max(phe)}/V_{max(tyr)}$	$(V/K_{phe})/(V/K_{tyr})$
TYH	6.8 ± 1.8	2.1 ± 0.2	0.31
PAH	>3	18.7 ± 2.0	<6.5
nP-TYH	12.1 ± 4.8	1.8 ± 0.04	0.15
nT-PAH	0.89 ± 0.20	10.4 ± 0.18	11.8
Δ155TYH	0.97 ± 0.20	1.8 ± 0.13	1.8
Δ117PAH	4.33 ± 1.0	10.5 ± 1.4	2.4

[a] Determined from the rate of 6-methyltetrahydropterin oxidation with 80 mM HEPES, 5−300 μM tyrosine, 60 μg/mL catalase, 150 μM 6-methyltetrahydropterin, 200 μM NADH, and 50 milliunits of DHPR, pH 7.1, 25 °C.

ties of PAH and TYH, we have focused on three aspects of the interactions with amino acid substrates. These are the steady-state kinetic parameters with phenylalanine and tyrosine as substrates, the need for activation by phenylalanine, and the coupling of tetrahydropterin oxidation and amino acid hydroxylation. In the following paragraphs, we will describe in detail the data which led to these main conclusions: (1) the substrate specificity for each C domain is determined by the C domain; (2) attachment of either R domain to either C domain enhances the substrate specificity determined by the C domain; and (3) only if the R domain of PAH is attached to the C domain of PAH does the hydroxylase require pretreatment with phenylalanine for full activity with the physiological cofactor.

Scheme 1 shows an alignment of the amino acid sequences of the amino-terminal halves of rat PAH and TYH. Based on the frequency of conserved residues, there is a clear region of homology which begins at valine 164 of TYH and valine 118 of PAH. The homology continues until the last 20 amino acids of these proteins. We selected the conserved sequence VPWFPR which contains this valine residue as the junction between the two domains. In addition to the sequence homology, the selection of this junction point is supported by studies of deletion mutants of these two enzymes. Mutants of rat TYH from which up to 165 amino acids have been removed from the N-terminus retain some catalytic activity (*9*, *26*, *30*), while up to 142 residues can be deleted from rat PAH without complete loss of activity (*27*). These results would place the N-terminus of the C domain within a 23 amino acid region, within which VPWFPR is the most conserved sequence.

It is easiest to begin discussion with the two proteins which lack the R domains completely, Δ117PAH and Δ155TYH. Surprisingly, it would be difficult to define Δ155TYH as either a phenylalanine or a tyrosine hydroxylase simply based on the relative K_M, V/K, or V_{max} values for the two amino acids (Table 6). In fact, the K_{phe} and K_{tyr} values for this enzyme are indistinguishable, while the relative V/K and V_{max} values when 6-methyltetrahydropterin oxidation is measured show a slight preference for phenylalanine as substrate. The other truncate, Δ117PAH, shows a preference for phenylalanine over tyrosine of only 4.3- or 2.4-fold, depending on whether K_M values or V/K values are compared. Thus, neither truncate shows strong preference for tyrosine or phenylalanine by criteria which should primarily reflect binding specificity.

A clear substrate specificity becomes apparent only when one compares the ability to carry out the hydroxylation of amino acids. The V_{max} value for 6-methyltetrahydropterin oxidation by Δ117PAH is 10-fold greater with phenylalanine than with tyrosine. Moreover, while Δ117PAH does consume tetrahydropterin in the presence of tyrosine, no DOPA is formed (Table 5). Δ155TYH is about twice as active with tyrosine as a substrate as Δ117PAH if rates of 6-methyltetrahydropterin oxidation are compared. Δ155TYH is able to hydroxylate phenylalanine to form tyrosine, but a significant fraction of the tetrahydropterin is consumed unproductively (Table 4). Thus, the ability to hydroxylate tyrosine serves as a clear criterion for defining the substrate specificity of these enzymes.

Published data for the *C. violaceum* PAH compare well with those reported here for Δ117PAH (*7*). The bacterial enzyme, which is homologous to the C-terminal amino acids of rat PAH, has a K_{phe} value of 176 μM and a V_{max} value of about 400 min^{-1}. This agreement suggests that Δ117PAH does indeed represent a minimal PAH.

Comparison of the properties of wild-type PAH and TYH with those of Δ117PAH and Δ155TYH shows that attachment of the R domain does indeed affect the substrate specificity of these enzymes. The effect is primarily on binding specificity rather than on the rate of catalysis. Both the K_{phe} and the V/K_{phe} values for TYH are an order of magnitude different from the respective values for Δ155TYH, while the V_{max} values change less than 2-fold. The formation of tyrosine is slightly less coupled to 6-methyltetrahydropterin oxidation with the wild-type enzyme than with the isolated C domain. There are also small increases in the kinetic parameters with tyrosine as substrate. The effect of the R domain on PAH, reflected in the differences between PAH and Δ117PAH, is less striking, but the latter enzyme already shows a preference for phenylalanine. The V_{max} value with tyrosine as substrate decreases about 2-fold, but the kinetic parameters with phenylalanine as substrate show that the wild-type enzyme is also slightly less effective at tyrosine formation. The primary effect of attachment of the R domain to PAH is that the enzyme now requires treatment with phenylalanine for high activity when tetrahydrobiopterin is used as a substrate (Table 3). The need for such pretreatment of PAH has been well documented (*31*, *32*). It appears to involve a significant conformational change with no significant change in the environment of the active site iron (*5*). The interaction between the two domains in wild-type PAH has been described as inhibitory based upon the need for pretreatment (*33*), but the data presented here show that characterization to be oversimplified.

The chimeric proteins nP-TYH and nT-PAH were constructed and characterized to ascertain whether the R domain of one hydroxylase could change the properties of the other. By the primary criteria of specificity defined above, the ability to hydroxylate tyrosine to form DOPA, the answer is clearly negative. This is consistent with the ability to carry out this hydroxylation being a fundamental property of the active site within the C domain. Similarly, attachment of the R domain of PAH to the C domain of TYH does not confer on the resulting protein nP-TYH a requirement for pretreatment with phenylalanine. A reasonable explanation for this is that the interactions between these two domain in PAH involve amino acid residues from both the C and the R domains of the protein. In other words, a minimal model in which the two C domains are equivalent in their ability to interact with the R domains is ruled out.

Still, there are some effects of attachment of the R domains, in that the chimeric proteins are significantly

different from isolated C domains. Attachment of the R domain of TYH to the C domain of PAH to make nT-PAH actually increases the specificity of this enzyme for phenylalanine, as reflected in the changes both in relative K_M and in V/K values (Table 6). This effect is due to a combination of increased K_{tyr} values and decreased K_{phe} values. Indeed, nT-PAH is the most specific phenylalanine hydroxylase among the six proteins examined. Attachment of the R domain of TYH to PAH appears to result in a more restricted active site, generating an enzyme which can better distinguish between its substrate and its product. The effect is clearly less than that of attaching the R domain of PAH to the C domain of PAH, since this chimera still does not require activation by phenylalanine and is more active with tyrosine as substrate than wild-type PAH. The chimeric PAH does not require pretreatment with phenylalanine, which is not surprising, but it also has a lower K_{phe} value than the wild-type enzyme. This may again reflect a general loss of flexibility in the active site.

Attachment of the R domain of PAH to the C domain of TYH to make the chimeric protein nP-TYH similarly increases the specificity of that enzyme for tyrosine. This is clearly reflected in the change in the relative K_M and V/K values for these two substrates upon going from Δ155TYH to nP-TYH. Again, attachment of the R domain from the other protein appears to result in a less flexible active site, even though the requirement for treatment with phenylalanine is not transferred.

The three-dimensional structure of Δ155TYH has recently been determined (*34*). In general, the protein is composed of loosely packed helices, suggesting a great deal of conformational flexibility. The active site is a deep cleft lined by four helices. The N-terminus is located near the open face of the cleft, so that the regulatory domain is likely to be positioned near the active site in the native protein. In addtion, there are two loops which guard the active site; these may close down to protect the active site from solvent during catalysis. These structural observations are consistent with the proposed model of a conformationally flexible active site which can be modified by the regulatory domain.

The rate-limiting step in turnover for TYH and PAH is thought to be formation of the hydroxylating intermediate (*35*, *36*). These two enzymes thus appear to discriminate between tyrosine and phenylalanine in a catalytic step rather than in binding. It is perhaps not surprising that TYH has not evolved to strongly discriminate against phenylalanine as substrate. The role of TYH is to produce DOPA for the biosynthesis of catecholamine neurotransmitters. Formation of tyrosine would generate more of the substrate for the enzyme, but would not result in a diversion of amino acids into a separate metabolic pathway. In contrast, if PAH were to hydroxylate tyrosine to DOPA, it would both divert the amino acid from catabolism and generate a potent inhibitor of the enzyme (*31*). PAH must be able to accommodate tyrosine in its active site, however, since tyrosine is the product of the enzyme-catalyzed reaction.

This work has highlighted some of the kinetic differences between rat tyrosine hydroxylase and rat phenylalanine hydroxylase, in an attempt to pinpoint the structural sources of those differences. The proposal that the N-terminal regulatory domains are responsible for all differences between the two hydroxylases has been shown to be simplistic; the isolated C domains of TYH and PAH can be clearly distinguished based upon their abilities to hydroxylate tyrosine and phenylalanine. However, the R domains clearly influence the substrate specificities of PAH and TYH by affecting the relative affinities for the two substrates. The ability of either R domain to increase the substrate specificity of either hydroxylase was unexpected. Possible explanations for this phenomenon are that there is a significant structural similarity between the portions of the R domains which influence substrate specificity or that the increase in specificity is due to a general loss of conformational flexibility in the wild-type enzymes compared to the isolated C domains. The inability of the R domain of PAH to transfer the need for pretreatment with phenylalanine for full activity with tetrahydrobiopterin establishes that this phenomenon involves unique interactions involving both domains of PAH.

Both TYH and PAH are activated by phosphorylation at serine residues in the R domain. The mechanism of this activation in TYH is known; catecholamine inhibitors bind to the iron in the active site of TYH, and phosphorylation prevents this inhibition (*21*). Phosphorylation of PAH is thought to decrease the levels of phenylalanine needed for activation (*32*). Our plans for future study include work on the mechanisms of phosphorylation using these chimeric mutants.

ACKNOWLEDGMENT

A portion of this work was carried out during a sabbatical at the University of Wisconsin. The authors thank Dr. John Markley for his generosity during that time. We thank Dr. Ian Jennings of the Royal Children's Hospital, Australia, for the generous gift of plasmid pRPH1. We also thank the members of the lab of Dr. W. W. Cleland, University of Wisconsin, especially Mr. Lac Lee and Ms. Barbara Gerratana, for helpful discussions and for the use of spectrophotometers for kinetic analyses.

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BI9711137