

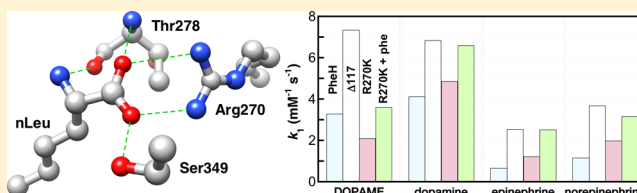
Activation of Phenylalanine Hydroxylase by Phenylalanine Does Not Require Binding in the Active Site

Kenneth M. Roberts, Crystal A. Khan, Cynthia S. Hinck, and Paul F. Fitzpatrick*

Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229, United States

Supporting Information

ABSTRACT: Phenylalanine hydroxylase (PheH), a liver enzyme that catalyzes the hydroxylation of excess phenylalanine in the diet to tyrosine, is activated by phenylalanine. The lack of activity at low levels of phenylalanine has been attributed to the N-terminus of the protein's regulatory domain acting as an inhibitory peptide by blocking substrate access to the active site. The location of the site at which phenylalanine binds to activate the enzyme is unknown, and both the active site in the catalytic domain and a separate site in the N-terminal regulatory domain have been proposed. Binding of catecholamines to the active-site iron was used to probe the accessibility of the active site. Removal of the regulatory domain increases the rate constants for association of several catecholamines with the wild-type enzyme by ~2-fold. Binding of phenylalanine in the active site is effectively abolished by mutating the active-site residue Arg270 to lysine. The $k_{\text{cat}}/K_{\text{phe}}$ value is down 10^4 for the mutant enzyme, and the K_{m} value for phenylalanine for the mutant enzyme is >0.5 M. Incubation of the R270K enzyme with phenylalanine also results in a 2-fold increase in the rate constants for catecholamine binding. The change in the tryptophan fluorescence emission spectrum seen in the wild-type enzyme upon activation by phenylalanine is also seen with the R270K mutant enzyme in the presence of phenylalanine. Both results establish that activation of PheH by phenylalanine does not require binding of the amino acid in the active site. This is consistent with a separate allosteric site, likely in the regulatory domain.



Phenylalanine hydroxylase (PheH) catalyzes the first step in the catabolism of excess phenylalanine in the diet, its hydroxylation to tyrosine. The enzyme activity in the liver responds in a cooperative fashion to phenylalanine concentrations, with low activity at basal concentrations of phenylalanine and increased activity when the concentration of phenylalanine in the blood rises.¹ The molecular basis for this behavior is not well-understood.² The initial rate of formation of tyrosine by purified PheH is very low unless the enzyme is first incubated with phenylalanine.³ The phenylalanine-activated enzyme displays positive cooperativity with phenylalanine as the substrate, with a Hill coefficient of ~2,⁴ consistent with additional cooperative interactions between different active sites. The enzyme can also be phosphorylated on Ser16; this decreases the concentration of phenylalanine required for activation.^{5,6} On the basis of extensive studies of the kinetics of activation of liver PheH under a variety of conditions, Shiman and co-workers⁷ proposed a model for the regulation of the enzyme. Key features of the model are that the resting form of the enzyme has little activity and that activation by phenylalanine is associated with a change in the active-site structure, allowing productive binding of substrates. The site at which phenylalanine binds to activate the enzyme was proposed to be distinct from the active site.⁸

The initial sequencing of PheH and the related aromatic amino acid hydroxylases tyrosine and tryptophan hydroxylase allowed the identification of two structural domains in these proteins, a homologous C-terminal catalytic domain and a

variable N-terminal domain.⁹ Subsequent analyses of mutant proteins established that the N-terminal domains are responsible for the divergent regulatory properties of the enzymes, while the C-terminal catalytic domains are responsible for substrate specificity^{10,11} and contain a C-terminal helix responsible for oligomerization.^{12–14} The crystal structure of a mutant form of rat PheH lacking the 24 C-terminal residues confirmed the presence of discrete regulatory and catalytic domains and provided a structural rationale for phenylalanine activation.^{15,16} In this structure, the N-terminus of the regulatory domain lies across the active site of the enzyme, partially occluding it (Figure 1). This observation led to the proposal that the conformational change that occurs upon activation by phenylalanine involves the displacement of this N-terminal tail, allowing access of substrates to the active site. Recently, Jaffe et al.¹⁷ proposed a structural model for the activated enzyme in which the regulatory domains dimerize.

There is no structure of PheH containing the regulatory domain that also has bound phenylalanine, leaving the location of the allosteric site unknown. Kobe et al.¹⁵ identified a possible site in the regulatory domain for phenylalanine based on their observation that the regulatory domain of PheH has a fold similar to that of the regulatory domain in 3-phosphoglycerate

Received: September 19, 2014

Revised: November 3, 2014

Published: November 24, 2014

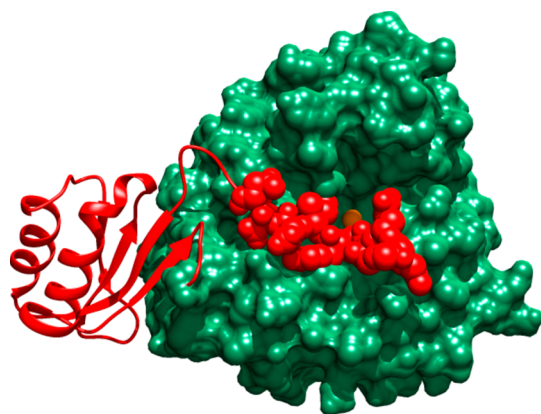


Figure 1. Crystal structure of the regulatory (red) and catalytic (green) domains of rat phenylalanine hydroxylase, based on Protein Data Bank entry 1PAH. The active-site iron is colored orange.

dehydrogenase that contains an allosteric site for serine.¹⁸ The same fold was subsequently identified in a number of other allosteric proteins and termed an ACT domain.^{19–21} More recently, Li et al.²² reported that addition of phenylalanine to the isolated regulatory domain of PheH altered the elution of the protein from a gel filtration column and changed the chemical shifts in a nuclear magnetic resonance heteronuclear single-quantum coherence spectrum of the protein, providing evidence of a phenylalanine binding site in the regulatory domain. Subsequently, Zhang et al.²³ reported that binding of phenylalanine to the isolated regulatory domain is linked to its dimerization, as predicted by the model proposed by Jaffe et al.¹⁷ In contrast, Thorolfsson et al.,²⁴ based on the application of calorimetry to intact and truncated proteins, concluded that phenylalanine activation involves binding only in the active site. Flydal et al.²⁵ reported that PheH from *Caenorhabditis elegans* has a Hill coefficient of 0.9 and is thus not cooperative but will bind two molecules of phenylalanine per subunit. Mutation of two residues in the catalytic domain of this protein to those found in human PheH decreased the stoichiometry of phenylalanine binding and increased the Hill coefficient to 1.4. These authors concluded that interactions with the catalytic domain prevent binding of phenylalanine to the regulatory domains of the mammalian enzymes and that this closure of the site in the regulatory domain is required for cooperativity.

Clearly, knowledge of the identity of any regulatory site for phenylalanine is critical to understanding the structural basis for regulation of PheH.² To address the question of whether activation of PheH by phenylalanine involves binding of the amino acid to an allosteric site outside of the catalytic site, we have now characterized the effects of binding of phenylalanine to a mutant PheH in which binding of the amino acid in the active site has been abolished. Because activation is proposed to be associated with increased access to the active site due to displacement of the N-terminus from the active site, we have used the kinetics of binding of catecholamines to the active-site iron as a measure of the accessibility of the active site. The global conformational change upon phenylalanine activation is accompanied by a change in the fluorescence emission of the enzyme, mainly due to altered solvent exposure of Trp120;²⁶ this has been used as a separate probe for activation.

EXPERIMENTAL PROCEDURES

Materials. BL21(DE3) cells were from Stratagene Corp. (La Jolla, CA). The pGro7 plasmid was from Takara Bio, Inc. (Otsu, Shiga, Japan). Pfu polymerase was from Agilent (Santa Clara, CA). Ampicillin was purchased from Research Products International Corp. (Mount Prospect, IL). Chloramphenicol and ferric chloride were from Acros Organics (Geel, Belgium). L-Arabinose was from Alfa Aesar (Ward Hill, MA). Magnesium sulfate and Tween 20 were from EMD Millipore Corp. (Billerica, MA). Isopropyl β -D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnology, Inc. (St. Louis, MO). Leupeptin was from Peptide Institute, Inc. (Ibaraki-Shi, Osaka, Japan). Lysozyme was from MP Biomedicals (Santa Ana, CA). Streptomycin sulfate was from Affymetrix, Inc. (Santa Clara, CA). Phenyl Sepharose CL-4B and Q Sepharose Fast Flow resins were from GE Healthcare Bio-Sciences (Pittsburgh, PA). L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride, epinephrine bitartrate, and norepinephrine bitartrate monohydrate were from Research Biochemicals, Inc. (Natick, MA). All other chemicals were from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO).

L-Phenylalanine obtained from all commercial sources examined contains trace amounts of L-tyrosine and other amino acids that interfered with the quantification of tyrosine formation by PheH Δ 117 R270K. Consequently, the amino acid was purified further by high-performance liquid chromatography (HPLC) on a C18 μ Bondapak (19 mm \times 300 mm) prep column using 2.5% methanol as the mobile phase, injecting 0.25 mL aliquots of 5 mg mL⁻¹ phenylalanine per run. The absorbance at 258 nm and fluorescence emission at 303 nm upon excitation at 275 nm were monitored simultaneously; the complete UV spectra of individual peaks were also recorded using an in-line diode array detector. The phenylalanine peaks eluting at \sim 23 min were collected; the eluents from multiple runs were pooled and concentrated to dryness on a rotary evaporator.

Protein Expression and Purification. A synthetic gene for wild-type rat PheH optimized for expression in *Escherichia coli* was obtained from DNA2.0 (Menlo Park, CA). The gene was amplified by polymerase chain reaction using oligonucleotides encoding the restriction sites for NcoI (5'-ATACCATGGGCGCTGTTGACTGGAA-3') and BamHI (5'-ATAGGATCCTTAGCTCTTAATCTTCTG-3'). This amplified gene was inserted into pET23d, and the resulting construct (pERPH2.0) was transformed into *E. coli* BL21(DE3) cells containing plasmid pGro7. The expression vector for the mutant PheH R270K was generated by mutagenesis of pERPH2.0 using Pfu polymerase and the QuikChange mutagenesis protocol. The oligonucleotides used as primers were 5'-CTGTACCCAATACATTAAGCACGGTAGCAAACCGATG-3', and 5'-CATCGGTTTGCTACCGTGCTTAATGTATTGGGTACAG-3'. The expression vector for PheH Δ 117 R270K was generated by mutagenesis of the plasmid pERPH Δ 117²⁷ with Pfu polymerase using the oligonucleotides 5'-CTGCACACAGTACATTAAGCATGGATCGAAGCCCATG-3' and 5'-CATGGGCTTCGATCCATGCTTAATGTACTGTGTGCAG-3' as primers. In all cases, the DNA sequence of the final plasmid used for expression was determined.

All protein purification steps were performed at 4 °C except for the chromatography, which was conducted at room temperature. The expression and purification of wild-type PheH were modified from those described by Daubner et al.²⁷

LB-Miller medium (100 mL) supplemented with 0.1 mg mL⁻¹ ampicillin, 35 µg mL⁻¹ chloramphenicol, and 0.5 mg mL⁻¹ L-arabinose was inoculated with one colony of BL21(DE3) pGro7 pERPH2.0. After the cells were grown overnight at 37 °C, Fernbach flasks (2.8 L) containing 2.0 L of LB-Miller medium, 4.0 mM magnesium sulfate, 1.0 mM ferric chloride, 0.1 mg mL⁻¹ ampicillin, 50 µg mL⁻¹ chloramphenicol, and 0.5 mg mL⁻¹ L-arabinose were inoculated with 14 mL each of the small culture. Cells were grown at 37 °C to an A₆₀₀ of 0.4; the temperature was then lowered to 18 °C. When the A₆₀₀ reached ~0.8, expression of the enzyme was induced with IPTG at a final concentration of 0.5 mM. After 21 h at 18 °C, cells were harvested by centrifugation at 7500g for 15 min at 4 °C. The cell pellets were stored at -80 °C. Frozen cell pellets were thawed and resuspended at 8 mL g⁻¹ in lysis buffer [30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mM L-phenylalanine, 200 mM NaCl, 0.2 mM diethylenetriaminepentaacetic acid (DTPA), 1 µM leupeptin, and 1 µM pepstatin A (pH 7.2)] containing 0.1 mg mL⁻¹ phenylmethanesulfonyl fluoride (PMSF) and 0.3 mg mL⁻¹ lysozyme and lysed by sonication. Nucleic acids were precipitated with 2% streptomycin sulfate. After centrifugation, the supernatant was stirred at room temperature for ~2 h and then loaded onto a Phenyl Sepharose CL-4B column (2.6 cm × 46 cm) equilibrated with lysis buffer. The column was washed with 2.5 column volumes of lysis buffer and then with 2.5 column volumes of lysis buffer containing 4.8% dimethylformamide and 0.1% Tween 20. PheH was eluted with 30 mM HEPES, 10–15% glycerol, 1 µM leupeptin, 1 µM pepstatin A, 50 µM DTPA, and 0.037% Tween 20 (pH 7.2). If the purity of the eluted protein was <90%, the protein was loaded onto a Q-Sepharose Fast-Flow column (1.6 cm × 57 cm) and washed with 250 mL of the same buffer. PheH was eluted with a gradient from 0 to 300 mM KCl in a total volume of 1.2 L of 30 mM HEPES and 10% glycerol (pH 7.2). The purified PheH was concentrated to ~30 mL using an Amicon stirred cell concentrator (EMD Millipore Corp.) with a 30 kDa NMWL filter. The iron content of the concentrated PheH was determined using the procedure of Roberts et al.²⁸ An amount of ferrous ammonium sulfate sufficient to result in a total iron concentration equivalent to one iron atom per monomer was added to the purified protein, and the mixture was stirred at 4 °C for 30 min. The protein was precipitated by addition of ammonium sulfate to 60% saturation, resuspended in 30 mL of 30 mM HEPES, 10–15% glycerol, 1 µM leupeptin, and 1 µM pepstatin A (pH 7.2), and then dialyzed against the same buffer for ~24 h.

PheH R270K was expressed and purified as described for wild-type PheH with minor modifications. Expression cultures were incubated at 18 °C throughout growth and expression, and cells were harvested ~26 h after induction. The expression and purification of PheH Δ117 were performed using the method of Roberts et al.²⁸

PheH Δ117 R270K was expressed with GroEL/ES in *E. coli* BL21(DE3) cells using the pERPHΔ117 R270K and pGro7 plasmids. One colony that had grown at 37 °C overnight on an LB agar plate containing 0.1 mg mL⁻¹ ampicillin and 34 µg mL⁻¹ chloramphenicol was used to inoculate 100 mL of LB medium containing 0.1 mg mL⁻¹ ampicillin, 0.1 mg mL⁻¹ chloramphenicol, and 0.5 mg mL⁻¹ L-arabinose. After growth overnight at 37 °C, 12 mL aliquots were used to inoculate six Fernbach flasks each containing 1 L of LB medium with 0.1 mg mL⁻¹ ampicillin, 0.1 mg mL⁻¹ chloramphenicol, and 0.5 mg mL⁻¹ L-arabinose. The cultures were allowed to grow at 37 °C

until the A₆₀₀ reached ~0.4, at which time the temperature was lowered to 20 °C. The cultures were induced with 60 mg L⁻¹ IPTG when they reached an A₆₀₀ of ~0.7. After 18 h at 20 °C, the cells were harvested by centrifugation at 7500g for 15 min. The cell pellets were resuspended at 8 mL g⁻¹ in 100 mM HEPES, 200 mM NaCl, 0.1 mM EDTA, 1 µM leupeptin, 1 µM pepstatin, 0.1 mg mL⁻¹ PMSF, and 0.1 mg mL⁻¹ lysozyme (pH 7.0). The lysate was passed through an 18 gauge needle until the lysate became homogeneous and then stirred for 20 min. The suspension was lysed by sonication. After centrifugation for 30 min at 15000g, streptomycin was added to the supernatant to a final concentration of 1%; after being stirred for 20 min, the solution was centrifuged for 30 min at 15000g. Ammonium sulfate was added to the supernatant to yield 40% saturation; after being stirred for 20 min, the solution was centrifuged for 30 min at 15000g. The precipitated protein was resuspended in resuspension buffer [100 mM HEPES, 0.1 mM EDTA, 15% glycerol, 1 µM leupeptin, and 1 µM pepstatin (pH 7.0)]. The protein was then dialyzed twice against resuspension buffer over 12 h. The dialyzed protein was centrifuged for 30 min at 15000g and then applied to a Q-Sepharose Fast-Flow column (1.6 cm × 57 cm) equilibrated with resuspension buffer. The column was washed with buffer until the A₂₈₀ was lower than 0.1. The protein was then eluted with a gradient from 0 to 250 mM NaCl in resuspension buffer. Fractions showing PheH at a purity of >90% by polyacrylamide gel electrophoresis were pooled and dialyzed against 100 mM HEPES, 0.1 mM EDTA, 15% glycerol, 1 µM leupeptin, and 1 µM pepstatin (pH 7.0), with three changes over 12 h. The protein was concentrated to 100–150 µM using an Amicon stirred cell concentrator containing a PM30 ultrafiltration membrane (EMD Millipore) and stored at -80 °C. Typically, 6 L yielded ~50 mg of purified protein.

Assays. Protein concentrations for the PheH R270K mutant proteins were determined using the extinction coefficients for wild-type PheH and PheH Δ117.²⁷ The phenylalanine hydroxylase activity of PheH Δ117 R270K was determined by HPLC on a Gemini-NX C18 150 mm × 2.0 mm column with a mobile phase of 0.1% acetic acid. The assays contained 3 µM enzyme, 0–100 mM purified L-phenylalanine, 250 µM 6-methyltetrahydropterin, 80 mM HEPES, 60 µg mL⁻¹ catalase, 1 mM dithiothreitol, and 5 µM ferrous ammonium sulfate (pH 7.0). These conditions were the same as those used previously for the wild-type enzyme,²⁷ although much lower concentrations of phenylalanine and enzyme were required in that case. Assays were initiated with enzyme and quenched at 0, 5, and 10 min with a 50% volume of 2 M HCl; in all cases, the reaction of the mutant enzyme was linear over this time period. The samples were then centrifuged for 10 min at 10000g and diluted 10-fold with 0.1% acetic acid before being injected onto the HPLC column. Tyrosine was detected by fluorescence with the excitation wavelength set at 275 nm and the emission wavelength set at 303 nm. A standard curve of 0–100 µM L-tyrosine was used to quantify the amount of tyrosine produced.

To determine the relative stoichiometry of tetrahydropterin oxidation and tyrosine formation, assays contained 10 or 20 µM enzyme, 100 µM 6-methyltetrahydropterin, 100 mM purified L-phenylalanine, and 80 mM HEPES (pH 7.0). Reactions were initiated by the addition of 6-methyltetrahydropterin and quenched after 5 min with a 50% volume of 2 M HCl. The amount of tyrosine produced was then determined by HPLC as described above.

The binding of catecholamines to PheH was monitored using an Applied Photophysics (Leatherhead, Surrey, U.K.) SX20 stopped-flow spectrophotometer. PheH (46 μM in iron) in 0.2 M HEPES (pH 7.0), with or without 2.0 mM L-phenylalanine, was mixed with varying concentrations of each catecholamine and the reaction followed at 700 nm. The binding of phenylalanine was monitored by a stopped-flow method with fluorescence detection, with an excitation wavelength of 295 nm and a cutoff filter at 345 nm; the path length of the cell was 10 mm, and the entrance and exit slit widths were set to 0.35 mm. Enzyme (5 μM) in 0.2 M HEPES (pH 7.5) in one syringe at 25 °C was mixed with 0.025–2 mM L-phenylalanine from the second. All concentrations are after mixing.

Data Analysis. Individual stopped-flow traces were analyzed as single and multiple exponentials using Kaleidagraph (Synergy Software, Reading, PA). Stopped-flow traces for the binding of multiple concentrations of each catecholamine to PheH were fit globally using KinTek Explorer.²⁹ In each fit, all rate constants, extinction coefficients, and y-axis offsets were allowed to vary. Confidence intervals for the best fit values for each rate constant were determined using the FitSpace module for KinTek Explorer.³⁰ The confidence intervals reported here are the values for each parameter that yield a sum square error that is 30% larger than that for the best fit when all other parameters are optimized.

RESULTS

Characterization of PheH R270K. In the active site of PheH, the carboxylate of the bound amino acid substrate forms ionic interactions with Arg270 and hydrogen bonds with the amide of Thr278 and the hydroxyl of Ser349 (Figure 2).

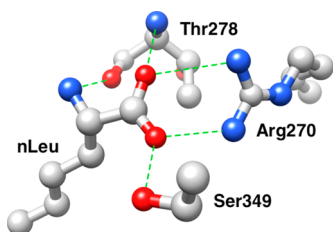


Figure 2. Amino acid substrate interactions in the active site of phenylalanine hydroxylase. The figure is based on the structure of the catalytic domain of human phenylalanine hydroxylase with norleucine (nLeu) and tetrahydrobiopterin (Protein Data Bank entry 1MMT).

Mutagenesis to lysine of the homologous residue in TyrH, Arg316, increases the K_m value for tyrosine to >10 mM and decreases the $k_{\text{cat}}/K_{\text{tyr}}$ value by 3700-fold.³¹ Consequently, Arg270 in PheH was mutated to lysine to decrease the level of binding of the amino acid substrate in the active site of that enzyme. The mutation was incorporated into both the intact protein and PheH $\Delta 117$, a mutant PheH that lacks the regulatory domain. The activity of the enzyme was measured with the latter, because it is fully active in the absence of activation by phenylalanine,²⁷ simplifying the kinetics. The mutant enzyme had no detectable activity at phenylalanine concentrations of <1 mM. When the activity was determined at phenylalanine concentrations from 5 to 100 mM, there was detectable activity that increased in a linear fashion with the phenylalanine concentration. This kinetic behavior sets a lower limit on the K_m value for phenylalanine of PheH R270K of 0.5 M and precludes measurement of a k_{cat} value. A $k_{\text{cat}}/K_{\text{phe}}$ of 1.7

$\pm 0.2 \text{ M}^{-1} \text{ min}^{-1}$ could be determined from these data. This value is down 10^6 -fold from the value for the wild-type enzyme.²⁷

Because mutagenesis of monooxygenases such as PheH frequently results in an uncoupling of hydroxylation and consumption of reducing equivalents, the relative stoichiometry of tetrahydropterin oxidation and tyrosine formation was determined for PheH R270K by determining the amount of tyrosine produced from a limiting amount of tetrahydropterin. Only $0.92 \pm 0.09\%$ of the pterin was oxidized to produce tyrosine; the rest was likely oxidized directly to dihydropterin.³²

Catecholamine Binding. The effect of activation by phenylalanine on the accessibility of the active site in PheH was analyzed by measuring the rate constants for binding of dopamine, norepinephrine, epinephrine, and 3,4-dihydroxyphenylalanine methyl ester (DOPAME) to the active-site iron for different forms of the enzyme. The complexes formed upon binding of the catechol oxygens to the iron are readily detected from the absorbance of a catecholate-to-metal charge transfer band near 700 nm.³³ Representative traces observed upon mixing the enzyme with dopamine are shown in Figure 3. For each catecholamine considered here, the traces for the wild-type enzyme could be fit ($R^2 > 0.99$) as single-exponential increases,^a with the rate constant for formation of the complex increasing linearly with the catecholamine concentration. To obtain the individual rate constants, the data for all concentrations of each catecholamine were fit globally with Scheme 1, which describes a simple reversible binding. The values for the second-order rate constant for association (k_1) range from 0.7 to 4 $\text{mM}^{-1} \text{ s}^{-1}$ for the different catecholamines (Table S1 of the Supporting Information and Figure 4). The k_1 values for epinephrine and norepinephrine are 3–6-fold lower than those for DOPAME and dopamine, indicating that the β -hydroxyl group results in slower binding of the catecholamine. The values for the rate constants for dissociation (k_{-1}) are small and are not significantly different from 0 s^{-1} in general; this is consistent with K_d values of <5 μM , in line with previous studies.^{34,35}

Mutant variants of PheH that lack the N-terminal regulatory domain do not require activation by phenylalanine.²⁷ In addition, the structures of the isolated catalytic domain of PheH with catecholamines bound show the catechol oxygens as ligands to the metal and the amine moiety exposed to solvent, so that catechols have unhindered access to the iron.³⁶ Consequently, the binding of catecholamines to a mutant PheH lacking the regulatory domain (PheH $\Delta 117$) was analyzed to determine the limiting rate constants for binding in the absence of any effect of the regulatory domain on access of ligands to the active site. The traces again could be described as single-exponential increases in absorbance, and the concentration dependencies were fit globally using the mechanism in Scheme 1 (Figure 3B). For all four catecholamines, the values for k_1 with PheH $\Delta 117$ were larger than those for the wild-type enzyme (Table S1 of the Supporting Information and Figure 4); the magnitude of the increase varied for each catecholamine. The value for dopamine showed the smallest change, increasing by 68%. The value for epinephrine experienced the largest change, increasing to nearly 4 times that with wild-type PheH. These increases are consistent with the absence of the regulatory domain resulting in increased accessibility of the active-site iron. The k_1 values for epinephrine and norepinephrine with PheH $\Delta 117$ were again significantly lower than those for DOPAME and dopamine,

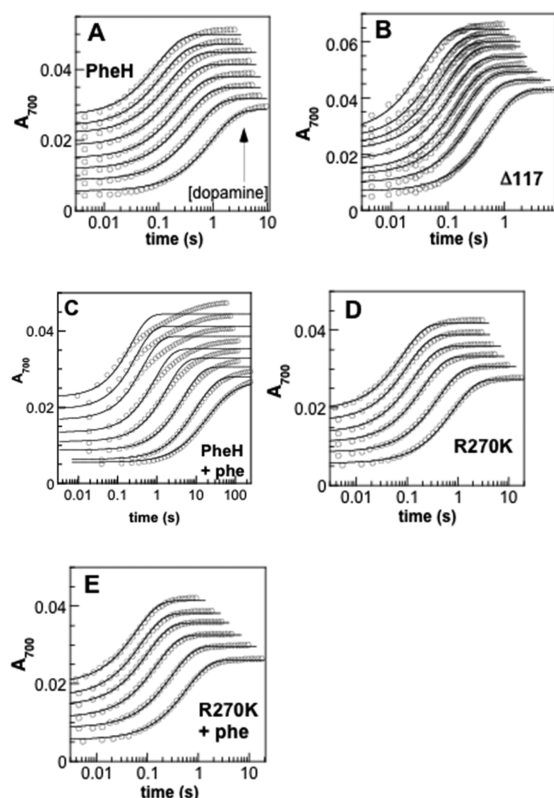


Figure 3. Stopped-flow traces for the binding of dopamine to the active-site iron of PheH. Reactions were performed by mixing 23 μM PheH with or without 2.0 mM L-phenylalanine with varying concentrations of dopamine (all concentrations are after mixing). The lines are from global fits of Scheme 1 for all traces within a given panel. The traces are offset, and only every third point is shown for the sake of clarity. (A) Wild-type PheH with 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, and 2.5 mM dopamine. (B) PheH $\Delta 117$ with 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, and 4.0 mM dopamine. (C) Wild-type PheH pre-mixed with phenylalanine and 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 mM dopamine. (D) PheH R270K with 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 μM dopamine. (E) PheH R270K pre-mixed with phenylalanine and 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 mM dopamine. Conditions: 200 mM HEPES, 25 μM DTPA, pH 7.0, 25 $^{\circ}\text{C}$.

Scheme 1

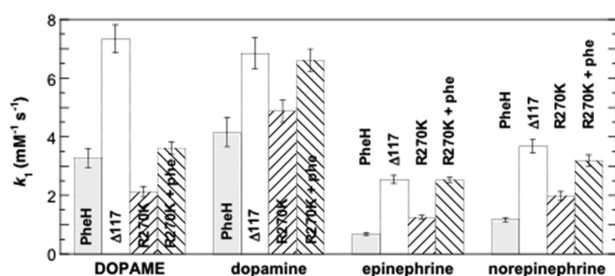
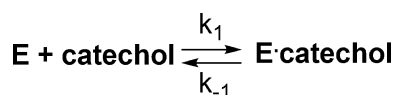


Figure 4. Rate constants for binding of catecholamines to PheH variants.

indicating that the effect seen with the wild-type enzyme is due to differences in binding to the catalytic domain and not the presence of the regulatory domain.

The proposal that the regulatory domain no longer blocks access to the active site in the phenylalanine-activated enzyme predicts that this form of the enzyme should bind catecholamines like PheH $\Delta 117$. However, the absorbance traces resulting from mixing phenylalanine-activated wild-type PheH with DOPAME or dopamine were markedly slower and more complex than in the absence of phenylalanine, fitting poorly to single exponentials with at least two phases visible at the higher catecholamine concentrations (Figure 3C and Figure S1 of the Supporting Information).

One possible explanation for the complex binding kinetics of the activated wild-type enzyme is that binding of phenylalanine in the active site is affecting catecholamine binding. Because phenylalanine binds in the active site of PheH R270K much more weakly than in the case of the wild-type enzyme, any effect of phenylalanine on binding of catecholamine to this mutant protein should reflect only changes due to binding of the amino acid at a site other than the active site. As is the case with the wild-type enzyme in the absence of phenylalanine, the kinetics of binding of all four catecholamines to the mutant protein were monophasic (Figure 3D), allowing the data to be analyzed using the mechanism of Scheme 1. The resulting kinetic constants are given in Figure 4 and Table S1 of the Supporting Information. All of the k_1 values are within 2-fold of the values for the wild-type enzyme and significantly lower than the values for PheH $\Delta 117$.

The catecholamine binding kinetics for PheH R270K in the presence of phenylalanine were much simpler than those of the wild-type enzyme in the presence of the amino acid, resembling instead those of PheH $\Delta 117$ and the unactivated wild-type enzyme (Figure 3E). For all four catecholamines, the values for k_1 with phenylalanine-treated PheH R270K were larger than the respective values in the absence of phenylalanine, and the increase in the rate constant for the mutant protein was comparable to that seen upon removing the regulatory domain from the wild-type enzyme (Figure 4). Indeed, for all but DOPAME, the values were within error of the k_1 values for PheH $\Delta 117$. Thus, binding of phenylalanine to PheH R270K makes the active site as accessible as complete removal of the regulatory domain. In light of the effect of this mutation on phenylalanine binding in the active site, this increased accessibility requires that there be a separate site for phenylalanine.

Stopped-Flow Fluorescence Spectroscopy of Phenylalanine Binding. Activation of PheH by phenylalanine is accompanied by a change in the fluorescence emission spectrum of the protein, which shifts to longer wavelengths.^{37,38} This allows the conformational change to be monitored by stopped-flow fluorescence spectroscopy. As shown in Figure 5A, mixing of either wild-type PheH or PheH R270K with 250 μM phenylalanine results in a relatively slow increase in the fluorescence emission at wavelengths greater than 345 nm. At this concentration of phenylalanine, the magnitudes of the fluorescence change are comparable for the two enzymes, although the change occurs ~ 5 -fold faster for the wild-type enzyme. The observation of this fluorescence change in a mutant protein unable to bind phenylalanine in the active site establishes that a protein conformational change consistent with activation involves binding at a site other than the active site. Analysis of the magnitude of the fluorescence change as a function of the concentration of phenylalanine showed that the behavior of the wild-type enzyme is more complex than that of the mutant protein, with the apparent binding of a second

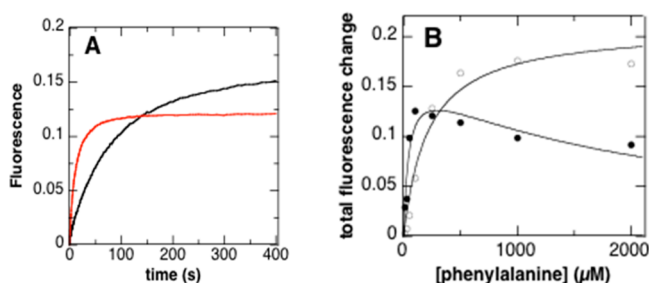


Figure 5. Fluorescence changes upon binding of phenylalanine to PheH. (A) Time course of the change in fluorescence during the binding of 250 μM phenylalanine to 5 μM wild-type PheH (red) or PheH R270K (black). (B) Total fluorescence change as a function of the phenylalanine concentration for wild-type PheH (●) and PheH R270K (○). The excitation wavelength was 295 nm, and the emitted light was passed through a 345 nm cutoff filter. The lines in panel B are from fits to $\Delta F = \Delta F_{\text{tot}}[\text{phenylalanine}]/(K_1 + [\text{phenylalanine}] + [\text{phenylalanine}]^2/K_2)$ for the wild-type enzyme and to $\Delta F = \Delta F_{\text{tot}}[\text{phenylalanine}]/(K_d + [\text{phenylalanine}])$ for the mutant enzyme. Conditions: 0.2 M HEPES, pH 7.5, 25 $^{\circ}\text{C}$.

phenylalanine resulting in a decrease in the total fluorescence change (Figure 5B).

DISCUSSION

The results of the experiments described here strongly support a model in which activation of PheH by phenylalanine involves binding at a site other than the active site. In light of the evidence that phenylalanine will bind the isolated regulatory domain,^{22,23} the most likely location for this allosteric site is in the regulatory domain.

Mutagenesis of Arg270 of PheH decreases the level of binding of the amino acid substrate in the active site to negligible levels at accessible phenylalanine concentrations. This is consistent with the ionic interaction between the carboxylate of the substrate and the side chain of Arg270 being critical for binding. The decrease in affinity calculated from the decrease in the $k_{\text{cat}}/K_{\text{phe}}$ value is 10^6 -fold, while the increase in the K_{m} value sets a lower limit of 3500-fold.²⁷ The $k_{\text{cat}}/K_{\text{phe}}$ value measured from tyrosine formation reflects only the turnovers that result in the formation of tyrosine, so that it will be decreased by unproductive oxidation of the tetrahydropterin substrate. Such uncoupling has been described previously for a number of mutants of PheH and the other aromatic amino acid hydroxylases.^{39–42} Unproductive enzyme-catalyzed tetrahydropterin oxidation still requires binding of both the pterin and the amino acid substrate before a reaction with oxygen occurs. It is the unproductive breakdown of an oxygen-containing intermediate prior to hydroxylation of the amino acid that results in the unproductive turnover.^{39,43,44} Correction of the $k_{\text{cat}}/K_{\text{phe}}$ value for PheH R270K for its 99% unproductive turnover yields a decrease of 12000-fold from that of the wild-type enzyme. This is comparable to the 3700-fold decrease in the $k_{\text{cat}}/K_{\text{m}}$ value for tyrosine when the identical mutation is incorporated into tyrosine hydroxylase.

No structure of PheH that has been activated by phenylalanine is yet available, so that the details of the structural changes accompanying activation are not known. The proposal that activation is a consequence of the movement of the N-terminus of the regulatory domain away from the active site¹⁵ predicts that molecules that bind the active site should do so more readily with the activated enzyme. We selected binding of

catecholamine to the active-site iron to test this prediction. The primary interaction of catecholamines with PheH is with the active-site iron³⁶ rather than with active-site residues, so that binding is less likely to be perturbed by any change within the active site than binding of a pterin or amino acid analogue would be. In addition, the ligand-to-metal charge transfer absorbance at ~ 700 nm allows the binding of a catechol to the iron to be readily monitored. The increase in the association rate constants for all four of the catecholamines analyzed here when the regulatory domain is absent supports a model in which activation involves increased active-site accessibility due to displacement of the N-terminal inhibitory peptide. Catecholamines do bind to the unactivated enzyme with significant rate constants, establishing that the iron is not completely inaccessible to solvent in the unactivated enzyme; this result is consistent with the known structure of PheH containing the regulatory domain (Figure 1). Critically, the magnitudes of the increases in the rate constants for formation of the catecholamine complexes by PheH R270K when phenylalanine is added agree well with the increases in these rate constants for the wild-type enzyme when the regulatory domain is removed. Thus, binding of phenylalanine at a site outside of the active site has the same effect on the accessibility of the active-site iron to catecholamines that complete removal of the regulatory domain does. This result provides substantive support for the proposed model of activation. It also establishes that a conformational change that makes the active site more accessible involves phenylalanine binding at a site separate from the active site.

A number of lines of evidence have established that the conformational change accompanying activation of PheH involves changes more extensive than simple displacement of an inhibitory peptide from the active site. The activated enzyme has a higher affinity for hydrophobic surfaces, a feature used in the most common purification protocol for PheH,⁴⁵ so that activation changes the exposure of hydrophobic residues on the protein surface. There is a significant change in the protein fluorescence due mainly to changes in the fluorescence emission maximum of Trp120.^{26,37} This residue is distant from the active site but close to the interface between the catalytic and regulatory domains, so that this interface is altered in the activated protein. A more detailed analysis of the structural change accompanying activation from mass spectrometric analysis of the effects of phenylalanine on the exchange of deuterium from solvent into peptide amides showed that the entire interface between the two domains incorporates deuterium more rapidly in the activated enzyme, not just the 30 N-terminal residues.⁴⁶ The proposal of Jaffe et al.¹⁷ provides a model for this change in the interactions between the two domains.

The change in tryptophan fluorescence emission when PheH is activated by phenylalanine was selected as a direct measure of this larger conformational change upon activation. That this fluorescence change is seen even with the R270K enzyme provides further evidence that phenylalanine activation is due to binding outside of the active site. Thus, binding of the amino acid to a site outside of the active site is associated with both the local change that exposes the active site and the more global change in the protein conformation.

There are differences between wild-type PheH and the R270K protein in the effects of phenylalanine on both the binding of catecholamines and the fluorescence change. In both cases, the wild type exhibits more complex behavior; this is

likely due to binding of the amino acid in the enzyme active site. We have not attempted to develop a complete kinetic model for binding of catecholamine to the activated wild-type enzyme. A minimal model in which there is competition between binding of the catecholamine to the iron and binding of phenylalanine in the active site gives an improved fit to the data but still does not fully replicate the kinetics (results not shown). The decrease in the magnitude of the fluorescence change at higher concentrations of the amino acid suggests that binding at the active site may be associated with an additional change in the environment of Trp120. Fully activated PheH still exhibits a Hill coefficient of ~ 2 for phenylalanine,⁴⁷ consistent with interactions between the active sites of different subunits. Mutagenesis of Trp120 to phenylalanine significantly decreases the cooperativity of the activated enzyme,²⁶ suggesting that the conformation of the protein near this residue is linked to the homotropic activation involving active-site binding. Cys237 lies at the interface of the catalytic, regulatory, and oligomerization domains of the enzyme and is close to Arg68 in the regulatory domain. The R68V and C237D mutants do not require activation by phenylalanine but still have Hill coefficients that are not significantly different from the value for the wild-type enzyme,⁴⁸ providing further evidence of the linkage of subunit–subunit interactions to binding of the amino acid substrate in the active site.

The regulation of PheH is clearly complex. As proposed by Shiman,⁸ phenylalanine binding is required for formation of the active enzyme. This work establishes that the initial activation by phenylalanine involves binding at a site other than the active site. The evidence that the isolated regulatory domain will bind phenylalanine and the loss of allosteric properties when the regulatory domain is removed establish the regulatory domain as the likely site for the activating allosteric site.

■ ASSOCIATED CONTENT

■ Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: fitzpatrickp@uthscsa.edu. Phone: (210) 567-8264. Fax: (210) 567-8778.

Funding

This work was supported in part by National Institutes of Health Grant R01 GM098140.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PheH, phenylalanine hydroxylase; DOPAME, 3,4-dihydroxyphenylalanine methyl ester; IPTG, isopropyl β -1-thiogalactopyranoside; PheH Δ 117, PheH lacking the 117 N-terminal residues; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; PMSF, phenylmethanesulfonyl fluoride.

■ ADDITIONAL NOTE

^aThere is a much slower increase in absorbance at longer times visible in several of the traces; this was ascribed to the slow oxidation of catecholamines and was not included in the kinetic analyses. Possibly as a result of this slower increase, fitting the

data to two exponentials rather than one resulted in a statistical improvement in the fitting in several cases (from $R^2 > 0.99$ to $R^2 > 0.999$). However, the trends seen in Figure 4 were unaffected.

■ REFERENCES

- (1) Shiman, R., Mortimore, G. E., Schworer, C. M., and Gray, D. W. (1982) Regulation of phenylalanine hydroxylase activity by phenylalanine *in vivo*, *in vitro*, and in perfused rat liver. *J. Biol. Chem.* 257, 11213–11216.
- (2) Fitzpatrick, P. F. (2012) Allosteric regulation of phenylalanine hydroxylase. *Arch. Biochem. Biophys.* 519, 194–201.
- (3) Shiman, R., and Gray, D. W. (1980) Substrate activation of phenylalanine hydroxylase. A kinetic characterization. *J. Biol. Chem.* 255, 4793–4800.
- (4) Knappskog, P. M., Flatmark, T., Aarden, J. M., Haavik, J., and Martinez, A. (1996) Structure/function relationships in human phenylalanine hydroxylase. Effect of terminal deletions on the oligomerization, activation and cooperativity of substrate binding to the enzyme. *Eur. J. Biochem.* 242, 813–821.
- (5) Abita, J. P., Milstien, S., Chang, N., and Kaufman, S. (1976) *In vitro* activation of rat liver phenylalanine hydroxylase by phosphorylation. *J. Biol. Chem.* 251, 5310–5314.
- (6) Doskeland, A. P., Martinez, A., Knappskog, P. M., and Flatmark, T. (1996) Phosphorylation of recombinant human phenylalanine hydroxylase: Effect on catalytic activity, substrate activation and protection against non-specific cleavage of the fusion protein by restriction protease. *Biochem. J.* 313, 409–414.
- (7) Shiman, R., Xia, T., Hill, M. A., and Gray, D. W. (1994) Regulation of rat liver phenylalanine hydroxylase. II. Substrate binding and the role of activation in the control of enzymatic activity. *J. Biol. Chem.* 269, 24647–24656.
- (8) Shiman, R., Jones, S. H., and Gray, D. W. (1990) Mechanism of phenylalanine regulation of phenylalanine hydroxylase. *J. Biol. Chem.* 265, 11633–11642.
- (9) Grenett, H. E., Ledley, F. D., Reed, L. L., and Woo, S. L. C. (1987) Full-length cDNA for rabbit tryptophan hydroxylase: Functional domains and evolution of aromatic amino acid hydroxylases. *Proc. Natl. Acad. Sci. U.S.A.* 84, 5530–5534.
- (10) Daubner, S. C., Lohse, D. L., and Fitzpatrick, P. F. (1993) Expression and characterization of catalytic and regulatory domains of rat tyrosine hydroxylase. *Protein Sci.* 2, 1452–1460.
- (11) Daubner, S. C., Hillas, P. J., and Fitzpatrick, P. F. (1997) Expression and characterization of the catalytic domain of human phenylalanine hydroxylase. *Arch. Biochem. Biophys.* 348, 295–302.
- (12) Lohse, D. L., and Fitzpatrick, P. F. (1993) Identification of the intersubunit binding region in rat tyrosine hydroxylase. *Biochem. Biophys. Res. Commun.* 197, 1543–1548.
- (13) Dickson, P. W., Jennings, I. G., and Cotton, R. G. H. (1994) Delineation of the catalytic core of phenylalanine hydroxylase and identification of glutamate 286 as a critical residue for pterin function. *J. Biol. Chem.* 269, 20369–20375.
- (14) Yang, X.-J., and Kaufman, S. (1994) High-level expression and deletion mutagenesis of human tryptophan hydroxylase. *Proc. Natl. Acad. Sci. U.S.A.* 91, 6659–6663.
- (15) Kobe, B., Jennings, I. G., House, C. M., Michell, B. J., Goodwill, K. E., Santarsiero, B. D., Stevens, R. C., Cotton, R. G. H., and Kemp, B. E. (1999) Structural basis of autoregulation of phenylalanine hydroxylase. *Nat. Struct. Biol.* 6, 442–448.
- (16) Jennings, I. G., Teh, T., and Kobe, B. (2001) Essential role of the N-terminal autoregulatory sequence in the regulation of phenylalanine hydroxylase. *FEBS Lett.* 488, 196–200.
- (17) Jaffe, E. K., Stith, L., Lawrence, S. H., Andrade, M., and Dunbrack, R. L., Jr. (2013) A new model for allosteric regulation of phenylalanine hydroxylase: Implications for disease and therapeutics. *Arch. Biochem. Biophys.* 530, 73–82.

- (18) Schuller, D. J., Grant, G. A., and Banaszak, L. J. (1995) The allosteric ligand site in the V_{\max} -type cooperative enzyme phosphoglycerate dehydrogenase. *Nat. Struct. Biol.* 2, 69–76.
- (19) Aravind, L., and Koonin, E. V. (1999) Gleaning non-trivial structural, functional and evolutionary information about proteins by iterative database searches. *J. Mol. Biol.* 287, 1023–1040.
- (20) Chipman, D. M., and Shaanan, B. (2001) The ACT domain family. *Curr. Opin. Struct. Biol.* 11, 694–700.
- (21) Grant, G. A. (2006) The ACT domain: A small molecule binding domain and its role as a common regulatory element. *J. Biol. Chem.* 281, 33825–33829.
- (22) Li, J., Ilangoan, U., Daubner, S. C., Hinck, A. P., and Fitzpatrick, P. F. (2011) Direct evidence for a phenylalanine site in the regulatory domain of phenylalanine hydroxylase. *Arch. Biochem. Biophys.* 505, 250–255.
- (23) Zhang, S., Roberts, K. M., and Fitzpatrick, P. F. (2014) Phenylalanine binding is linked to dimerization of the regulatory domain of phenylalanine hydroxylase. *Biochemistry* 53, 6625–6627.
- (24) Thorolfsson, M., Ibarra-Molero, B., Fojan, P., Petersen, S. B., Sanchez-Ruiz, J. M., and Martinez, A. (2002) L-Phenylalanine binding and domain organization in human phenylalanine hydroxylase: A differential scanning calorimetry study. *Biochemistry* 41, 7573–7585.
- (25) Flydal, M. I., Mohn, T. C., Pey, A. L., Siltberg-Liberles, J., Teigen, K., and Martinez, A. (2010) Superstoichiometric binding of L-Phe to phenylalanine hydroxylase from *Caenorhabditis elegans*: Evolutionary implications. *Amino Acids* 39, 1463–1475.
- (26) Knappskog, P. M., and Haavik, J. (1995) Tryptophan fluorescence of human phenylalanine hydroxylase produced in *Escherichia coli*. *Biochemistry* 34, 11790–11799.
- (27) Daubner, S. C., Hillas, P. J., and Fitzpatrick, P. F. (1997) Characterization of chimeric pterin-dependent hydroxylases: Contributions of the regulatory domains of tyrosine and phenylalanine hydroxylase to substrate specificity. *Biochemistry* 36, 11574–11582.
- (28) Roberts, K. M., Pavon, J. A., and Fitzpatrick, P. F. (2013) Kinetic mechanism of phenylalanine hydroxylase: Intrinsic binding and rate constants from single-turnover experiments. *Biochemistry* 52, 1062–1073.
- (29) Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) Global Kinetic Explorer: A new computer program for dynamic simulation and fitting of kinetic data. *Anal. Biochem.* 387, 20–29.
- (30) Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) FitSpace Explorer: An algorithm to evaluate multidimensional parameter space in fitting kinetic data. *Anal. Biochem.* 387, 30–41.
- (31) Daubner, S. C., and Fitzpatrick, P. F. (1999) Site-directed mutants of charged residues in the active site of tyrosine hydroxylase. *Biochemistry* 38, 4448–4454.
- (32) Roberts, K. M., Tormos, J. R., and Fitzpatrick, P. F. (2014) Characterization of unstable products of flavin- and pterin-dependent enzymes by continuous-flow mass spectrometry. *Biochemistry* 53, 2672–2679.
- (33) Cox, D. D., Benkovic, S. J., Bloom, L. M., Bradley, F. C., Nelson, M. J., Que, L., Jr., and Wallick, D. E. (1988) Catecholate LMCT bands as probes for the active sites of nonheme iron oxygenases. *J. Am. Chem. Soc.* 110, 2026–2032.
- (34) Shiman, R., Gray, D. W., and Hill, M. A. (1994) Regulation of rat liver phenylalanine hydroxylase. I. Kinetic properties of the enzyme's iron and enzyme reduction site. *J. Biol. Chem.* 269, 24637–24646.
- (35) Martinez, A., Haavik, J., and Flatmark, T. (1990) Cooperative homotropic interaction of L-noradrenaline with the catalytic site of phenylalanine 4-monooxygenase. *Eur. J. Biochem.* 193, 211–219.
- (36) Erlandsen, H., Flatmark, T., Stevens, R. C., and Hough, E. (1998) Crystallographic analysis of the human phenylalanine hydroxylase catalytic domain with bound catechol inhibitors at 2.0 Å resolution. *Biochemistry* 37, 15638–15646.
- (37) Phillips, R. S., Parniak, M. A., and Kaufman, S. (1984) Spectroscopic investigation of ligand interaction with hepatic phenylalanine hydroxylase: Evidence for conformational change associated with activation. *Biochemistry* 23, 3836–3842.
- (38) Kappock, T. J., Harkins, P. C., Friedenber, S., and Caradonna, J. P. (1995) Spectroscopic and kinetic properties of unphosphorylated rat hepatic phenylalanine hydroxylase expressed in *Escherichia coli*. Comparison of resting and activated states. *J. Biol. Chem.* 270, 30532–30544.
- (39) Kemsley, J. N., Wasinger, E. C., Datta, S., Mitić, N., Acharya, T., Hedman, B., Caradonna, J. P., Hodgson, K. O., and Solomon, E. I. (2003) Spectroscopic and kinetic studies of PKU-inducing mutants of phenylalanine hydroxylase: Arg158Gln and Glu280Lys. *J. Am. Chem. Soc.* 125, 5677–5686.
- (40) Daubner, S. C., Avila, A., Bailey, J. O., Barrera, D., Bermudez, J. Y., Giles, D. H., Khan, C. A., Shaheen, N., Thompson, J. W., Vasquez, J., Oxley, S. P., and Fitzpatrick, P. F. (2013) Mutagenesis of a specificity-determining residue in tyrosine hydroxylase establishes that the enzyme is a robust phenylalanine hydroxylase but a fragile tyrosine hydroxylase. *Biochemistry* 52, 1446–1455.
- (41) Frantom, P. A., and Fitzpatrick, P. F. (2003) Uncoupled forms of tyrosine hydroxylase unmask kinetic isotope effects on chemical steps. *J. Am. Chem. Soc.* 125, 16190–16191.
- (42) Fitzpatrick, P. F. (2005) Isotope effects from partitioning of intermediates in enzyme-catalyzed hydroxylation reactions. In *Isotope Effects in Chemistry and Biology* (Kohen, A., and Limbach, H., Eds.) pp 861–873, Marcel Dekker, Inc., New York.
- (43) Chow, M. S., Eser, B. E., Wilson, S. A., Hodgson, K. O., Hedman, B., Fitzpatrick, P. F., and Solomon, E. I. (2009) Spectroscopy and kinetics of wild-type and mutant tyrosine hydroxylase: Mechanistic insight into O_2 activation. *J. Am. Chem. Soc.* 131, 7685–7698.
- (44) Krzyaniak, M. D., Eser, B. E., Ellis, H. R., Fitzpatrick, P. F., and McCracken, J. (2013) A pulsed EPR study of amino acid and tetrahydropterin binding in a tyrosine hydroxylase nitric oxide complex: Evidence for substrate rearrangements in formation of the oxygen-reactive complex. *Biochemistry* 52, 8430–8441.
- (45) Shiman, R., Gray, D. W., and Pater, A. (1979) A simple purification of phenylalanine hydroxylase by substrate-induced hydrophobic chromatography. *J. Biol. Chem.* 254, 11300–11306.
- (46) Li, J., Dangott, L. J., and Fitzpatrick, P. F. (2010) Regulation of phenylalanine hydroxylase: Conformational changes upon phenylalanine binding detected by hydrogen/deuterium exchange and mass spectrometry. *Biochemistry* 49, 3327–3335.
- (47) Shiman, R. (1980) Relationship between the substrate activation site and catalytic site of phenylalanine hydroxylase. *J. Biol. Chem.* 255, 10029–10032.
- (48) Thorolfsson, M., Teigen, K., and Martinez, A. (2003) Activation of phenylalanine hydroxylase: Effect of substitutions at Arg68 and Cys237. *Biochemistry* 42, 3419–3428.