

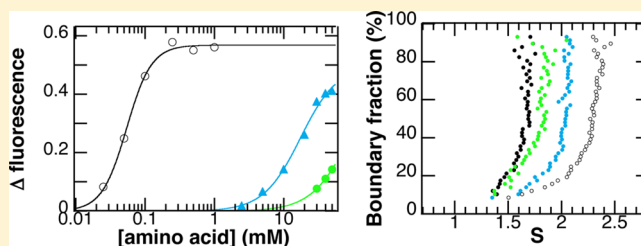
The Amino Acid Specificity for Activation of Phenylalanine Hydroxylase Matches the Specificity for Stabilization of Regulatory Domain Dimers

Shengnan Zhang, Andrew P. Hinck, and Paul F. Fitzpatrick*

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

S Supporting Information

ABSTRACT: Liver phenylalanine hydroxylase is allosterically activated by phenylalanine. The structural changes that accompany activation have not been identified, but recent studies of the effects of phenylalanine on the isolated regulatory domain of the enzyme support a model in which phenylalanine binding promotes regulatory domain dimerization. Such a model predicts that compounds that stabilize the regulatory domain dimer will also activate the enzyme. Nuclear magnetic resonance spectroscopy and analytical ultracentrifugation were used to determine the ability of different amino acids and phenylalanine analogues to stabilize the regulatory domain dimer. The abilities of these compounds to activate the enzyme were analyzed by measuring their effects on the fluorescence change that accompanies activation and on the activity directly. At concentrations of 10–50 mM, D-phenylalanine, L-methionine, L-norleucine, and (S)-2-amino-3-phenyl-1-propanol were able to activate the enzyme to the same extent as 1 mM L-phenylalanine. Lower levels of activation were seen with L-4-aminophenylalanine, L-leucine, L-isoleucine, and 3-phenylpropionate. The ability of these compounds to stabilize the regulatory domain dimer agreed with their ability to activate the enzyme. These results support a model in which allosteric activation of phenylalanine hydroxylase is linked to dimerization of regulatory domains.



Phenylalanine hydroxylase (PheH) belongs to the family of pterin-dependent aromatic amino acid hydroxylases, together with tyrosine hydroxylase and tryptophan hydroxylase.¹ Each enzyme catalyzes the hydroxylation of the aromatic side chain of its respective amino acid substrate, using tetrahydrobiopterin (BH₄) as the biological reductant and oxygen as the third substrate. All the eukaryotic hydroxylases form homotetramers; each monomer contains an N-terminal regulatory domain, a homologous catalytic domain, and a C-terminal tetramerization domain. The crystal structures of the catalytic domains confirm their very similar structures and active sites,^{2–4} consistent with these enzymes sharing a common catalytic mechanism.⁵ The structures of the regulatory domains of PheH and TyrH show that both contain ACT domains,^{2,6,7} although the two enzymes are regulated differently.^{8–11}

PheH catalyzes the hydroxylation of phenylalanine to tyrosine in the liver. A deficiency of human PheH increases the level of phenylalanine in the blood, resulting in the inherited disease phenylketonuria.¹² The activity of the enzyme must be tightly controlled, so that only excess phenylalanine is catabolized while leaving sufficient phenylalanine for protein synthesis. The present model for the regulation of PheH is based on experiments conducted with the rat enzyme, but the regulatory properties of human PheH are not significantly different.¹³ The enzyme has low activity unless it is preincubated with phenylalanine.¹⁴ The activated enzyme

displays positive cooperativity with respect to phenylalanine, with a Hill coefficient of 2–3.^{12,15} Binding of BH₄ to the unactivated enzyme prevents activation by phenylalanine.⁸ Phosphorylation of Ser16 also increases the activity of rat PheH, but less than activation by phenylalanine,¹⁶ and decreases the concentration of phenylalanine required to activate PheH.^{17,18}

The crystal structure of a dimeric form of unactivated rat PheH containing both the regulatory and catalytic domains showed that the N-terminus of the regulatory domain lies across the active site of the catalytic domain, likely preventing substrate binding.² Activation by phenylalanine was proposed to result in a conformational change, so that this portion of the regulatory domain no longer hinders access to the active site.¹⁹ However, there is as yet no available structure of PheH containing both the regulatory domain and bound phenylalanine, so that details of this conformational change are lacking. Indeed, the question of whether there is a phenylalanine binding site in the regulatory domain of PheH or whether activation involves binding only in the active site has been controversial.^{20–23} Recent studies have confirmed that phenylalanine does indeed bind to the isolated regulatory domain of PheH (RDPheH),^{24,25} but there is still disagreement

Received: June 4, 2015

Revised: August 6, 2015

Published: August 7, 2015

about whether this can occur in the context of the intact protein.¹² However, Roberts et al. have shown that elimination of phenylalanine binding in the active site does not prevent the conformational change associated with phenylalanine activation, consistent with an allosteric site separate from the active site.²⁶

Jaffe et al.²⁷ recently proposed a model for the activated form of PheH, in which two regulatory domains form an ACT-domain dimer, with phenylalanine binding at the dimer interface. This behavior is consistent with the known properties of ACT domains, which often act as allosteric modules that oligomerize in response to ligand binding.^{7,28} This model is supported by our findings that RDPheH exists in a monomer-dimer equilibrium in solution and that phenylalanine binding stabilizes the dimer²⁴ and by the formation of a stable ACT-domain dimer by the regulatory domain of tyrosine hydroxylase.⁶ Still, there is no direct evidence that the phenylalanine binding results in dimerization of the regulatory domains in intact PheH. If activation of the enzyme by phenylalanine is indeed linked to dimerization of the regulatory domains, compounds that stabilize the RDPheH dimer should also activate PheH. We report here that besides its natural substrate phenylalanine, a number of other amino acids and phenylalanine analogues stabilize the RDPheH dimer. In addition, the abilities of these compounds to stabilize the RDPheH dimer agree with their abilities to activate PheH. These results support a model in which activation of PheH by phenylalanine is linked to dimerization of the regulatory domain.

EXPERIMENTAL PROCEDURES

Materials. ¹⁵NH₄Cl was from Cambridge Isotope Laboratories, Inc. (Andover, MA). BH₄ was purchased from Schircks Laboratories (Jona, Switzerland). Dithiothreitol was from Inalco, S.p.A. (Milan, Italy). Leupeptin and pepstatin A were from Peptide Institute, Inc. (Osaka, Japan). L-Norleucine was purchased from MP Biomedicals, Inc. (Solon, OH). All the other amino acids and phenylalanine analogues were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Protein Expression and Purification. The expression and purification of rat RDPheH and the N-terminal 24-residue deletion mutant (RDPheH_{25–117}) were performed as previously described.^{24,25} For ¹⁵N-labeled RDPheH and RDPheH_{25–117}, the expression and purification were the same as for the unlabeled proteins, except that the cells were grown in M9 minimal medium with ¹⁵NH₄Cl (1 g/L).²⁹ The expression and purification of wild-type rat PheH were performed as previously described.^{26,30} The purities of all protein preparations were >95% based on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Nuclear Magnetic Resonance (NMR) Spectroscopy. ¹H–¹⁵N HSQC spectra were routinely collected at 300 K on a Bruker Avance 600 spectrometer using a 5 mm TXI (¹H/¹³C/¹⁵N) CryoProbe with z-axis pulsed field gradients. NMR samples were prepared in buffer A [50 mM phosphate, 100 mM NaCl, 1 μM leupeptin, and 1 μM pepstatin A (pH 8)] and 5% D₂O. A pH of 8 was selected despite the loss of some signals because both RDPheH and RDPheH_{25–117} precipitate too rapidly at pH <8 for NMR analyses, consistent with a calculated pI value of 6.8. NMR screening for formation of the RDPheH_{25–117} dimer was performed at 300 K on a Bruker Avance 500 spectrometer equipped with a SampleJet sample changer and a 1.7 mm TCI (¹H/¹³C/¹⁵N) Micro-CryoProbe.

NMR samples for screening were made using a Gilson 215 Liquid Handler. ¹⁵N-labeled RDPheH_{25–117} (20 μL of 800 μM monomer in buffer A with 20% D₂O) was mixed with an equal volume of a solution of the compound of interest [20 or 100 mM in buffer A (pH 8)], and 36 μL of the mixture was transferred to a 1.7 mm Sample Jet tube. All spectra were processed using NMRPipe³¹ and analyzed using NMRView.³²

Analytical Ultracentrifugation. The effects of potential activators on the dimerization of RDPheH were determined by analytical ultracentrifugation (AUC) as previously described.²⁴ Sedimentation velocity experiments were conducted using RDPheH_{25–117} (~15 μM total monomer) with detection at 230 nm. AUC samples were prepared in 50 mM phosphate and 100 mM NaCl (pH 8.0). Ultrascan III³³ was used for van Holde–Weischet analyses of the AUC data. The standard *c(s)* model of SEDFIT³⁴ version 14.1 was used to generate *c(s)* distributions. The values for the weighted-average sedimentation coefficient (*s_w*) were determined by integration of the *c(s)* distribution between 1 and 3 S.

Fluorescence Spectroscopy. Binding to the allosteric site in PheH was monitored as previously described for phenylalanine binding.²⁶ PheH [10 μM in 0.2 M HEPES (pH 7.5)] in one syringe of an Applied Photophysics (Leatherhead, Surrey, U.K.) SX18 stopped-flow spectrofluorometer was mixed with an equal volume of a 10–100 mM solution of each compound in the same buffer from the other syringe at 25 °C. The intrinsic tryptophan fluorescence of the protein was monitored using excitation at 295 nm and an emission cutoff filter of 340 nm. The reaction was followed until no further fluorescence changes occurred, typically 2–5 min.

Enzyme Assays. The effect of preincubation with different compounds on the activity of PheH was based on methods used previously to demonstrate phenylalanine activation.³⁰ PheH [10 μL of 50 μM in 200 mM HEPES (pH 7.0)] was mixed with an equal volume of each compound (20 or 100 mM) or 2 mM phenylalanine in the same buffer at 23 °C; after 10 min, 5 μL of the mixture was added to 495 μL containing all assay components [1 mM phenylalanine, 200 μM BH₄, 50 μg/mL catalase, 1 mM dithiothreitol, 5 μM ferrous ammonium sulfate, and 80 mM HEPES (pH 7.0)]. The assay was quenched with 250 μL of 2 M HCl after 30 s and centrifuged for 5 min at 10000g. After 10-fold dilution with 0.1% acetic acid, the samples were loaded onto a Gemini-NX C18 (150 mm × 2.0 mm) HPLC column with 0.1% acetic acid as the mobile phase. Tyrosine was detected by fluorescence with the excitation wavelength set at 275 nm and the emission wavelength set at 303 nm.

RESULTS

NMR Spectroscopy of RDPheH. Figure 1A shows the two-dimensional (2D) ¹H–¹⁵N HSQC spectrum of 1 mM RDPheH at pH 8.0. Our previous studies of RDPheH established that it is a folded protein that can bind phenylalanine.²⁵ The cluster of signals with high intensity in the random-coil region suggests that a portion of the protein is disordered. This is consistent with the crystal structure of rat PheH containing only the regulatory and catalytic domains, which lacks electron density for the 18 N-terminal residues.² To simplify the spectrum, a series of variants of RDPheH lacking residues in the disordered N-terminal tail were examined as NMR samples. The mutant protein lacking the 24 N-terminal residues (RDPheH_{25–117}) was more stable than full-length RDPheH, with a lower propensity to precipitate at NMR

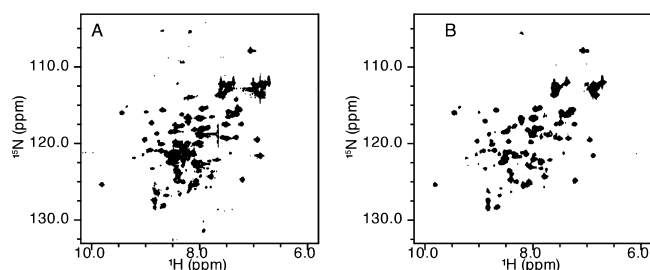


Figure 1. Effect of N-terminal deletion on the ^1H – ^{15}N HSQC NMR spectrum of the regulatory domain of PheH: (A) 1 mM RDPHeH and (B) 430 μM RDPHeH_{25–117}. Conditions: 50 mM sodium phosphate, 100 mM NaCl, 1 μM leupeptin, 1 μM pepstatin A, and 5% D₂O (pH 8.0), at 300 K at a magnetic field strength of 14.1 T (600 MHz for ^1H).

concentrations. This truncation also eliminated most of the high-intensity signals in the random-coil region but left the more dispersed lower-intensity signals unperturbed (Figure 1B), indicating that the 24 N-terminal residues are flexible and not necessary for the core structure of the regulatory domain. We have previously shown that removal of the 24 N-terminal residues has no effect on the dimerization or phenylalanine binding of RDPHeH.²⁴

Figure 2A shows the ^1H – ^{15}N HSQC spectrum of 3 mM RDPHeH_{25–117} at pH 8.0. On the basis of the previously

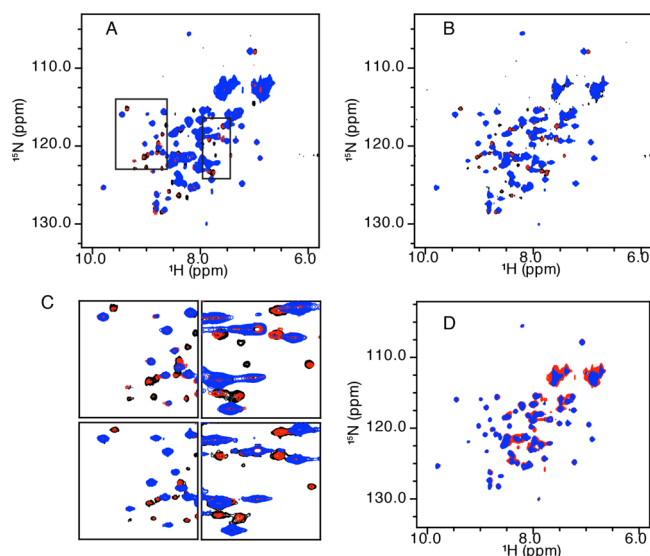


Figure 2. Effects of the concentrations of protein and phenylalanine on the NMR spectrum of the isolated regulatory domain of PheH. (A) Overlay of the 2D ^1H – ^{15}N HSQC spectra of RDPHeH_{25–117} at 50 μM (black), 800 μM (red), and 3 mM (blue). (B) Overlay of the 2D ^1H – ^{15}N HSQC spectra of 480 μM RDPHeH_{25–117} alone (black) and with 100 μM (red) or 2 mM (blue) phenylalanine. (C) Expansion of the regions in panels A and B indicated by boxes in panel A. The top two rectangles are from panel A while the bottom two from panel B. (D) Overlay of the 2D ^1H – ^{15}N HSQC spectra of 3 mM RDPHeH_{25–117} (red) and 480 μM RDPHeH_{25–117} and 2 mM phenylalanine (blue).

reported dissociation constant for dimerization (46 μM),²⁴ this spectrum should be essentially that of the dimer. Consistent with this expectation, ~ 75 cross-backbone amide signals can be identified in the spectrum, compared to the 91 anticipated for RDPHeH_{25–117}. (Several additional resonances could be

detected at pH 7.0, but the protein is too poorly soluble below pH 8.0 for NMR analyses.) At lower protein concentrations, additional cross-peaks are observed (Figure 2A); their intensities increase with a decrease in protein concentration, while the intensities of some of the dimer peaks decrease. These intensity changes with protein concentrations are consistent with RDPHeH_{25–117} existing in a monomer–dimer equilibrium in solution. The equilibrium must be in relatively slow exchange on the NMR chemical shift time scale, because no peaks with intermediate chemical shifts are seen. The variability in the intensities of resonances is probably due mostly to rapid solvent exchange at the high pH, but some may also be due to monomer and dimer residues in the intermediate exchange regime. To date, we have been able to assign 64 of the backbone amide resonances to single residues (S. Zhang, and P. F. Fitzpatrick, unpublished observations). This is consistent with RDPHeH dimer being a symmetric dimer.

Figure 2B shows the spectral changes observed upon titration of 480 μM RDPHeH_{25–117} ($\sim 80\%$ dimer) with phenylalanine; the changes are similar to those in the NMR spectrum of intact RDPHeH described previously.²⁵ The changes are also very similar to those seen with an increase in protein concentration (Figure 2A,C), with the intensities of the cross-peaks due to the monomer decreasing with an increase in phenylalanine concentration. This result confirms that phenylalanine binds to the dimeric form of RDPHeH_{25–117}, consistent with our previous model for phenylalanine binding.²⁴ The HSQC spectrum of 3 mM RDPHeH_{25–117} and that of 430 μM RDPHeH_{25–117} with 1 mM phenylalanine are essentially the same (Figure 2D), indicating that phenylalanine binding does not alter the backbone structure of RDPHeH.

Stabilization of the RDPHeH Dimer. The differences in the NMR spectra of the monomeric and dimeric forms of RDPHeH were used to identify compounds that can stabilize the RDPHeH dimer. A series of HSQC spectra of 400 μM RDPHeH_{25–117} with amino acids and phenylalanine analogues were collected and compared with that of protein alone. Most of the standard L-amino acids were screened; cysteine, tyrosine, and tryptophan were not selected because of their poor solubilities at pH 8. Spectral changes similar to those seen in the presence of L-phenylalanine (Figure 2C) were observed when RDPHeH_{25–117} was mixed with D-phenylalanine, L-4-aminophenylalanine, L-norleucine, or L-methionine at a concentration of 10 mM (Figure 3 and Figure S1). Addition of the three branched-chain L-amino acids also caused significant spectral changes when their concentrations were increased to 50 mM (Figure S1). The interaction between L-valine and RDPHeH is the weakest, in that there were still some monomer peaks in the HSQC spectrum of 400 μM RDPHeH_{25–117} in the presence of 50 mM valine. Addition of 10 mM (S)-2-amino-3-phenyl-1-propanol or 50 mM 3-phenylpropionate resulted in several chemical shift changes in the HSQC spectra in addition to the intensity changes (Figure S1E,F), generally in residues whose intensities increased in the presence of phenylalanine. The other potential ligands did not cause any detectable spectral changes even at 50 mM. These data suggest that D-phenylalanine, L-4-aminophenylalanine, (S)-2-amino-3-phenyl-1-propanol, 3-phenylpropionate, L-norleucine, L-methionine, L-leucine, L-isoleucine, and possibly L-valine can stabilize the RDPHeH dimer, although with affinities much lower than that of phenylalanine.

The ability of these compounds to stabilize the RDPHeH dimer was determined directly using sedimentation velocity

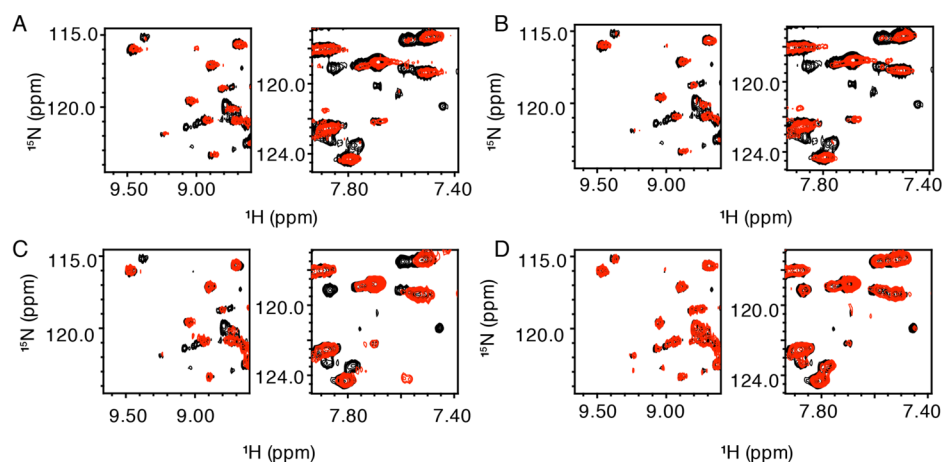


Figure 3. Effects of selected amino acids on the 2D ^1H – ^{15}N HSQC spectra of RDPHeH $_{25-117}$: 400 μM RDPHeH $_{25-117}$ (black) and 400 μM RDPHeH $_{25-117}$ (red) with (A) 10 mM D-phenylalanine, (B) 10 mM methionine, (C) 10 mM L-4-aminophenylalanine, and (D) 50 mM alanine. The two regions of the spectra shown are the same as those in Figure 2C. Conditions: 50 mM sodium phosphate, 100 mM NaCl, 1 μM leupeptin, 1 μM pepstatin A, and 10% D $_2\text{O}$ (pH 8.0), at 300 K at a magnetic field strength of 11.7 T (500 MHz for ^1H).

ultracentrifugation. Methionine, 3-phenylpropionate, and L-4-aminophenylalanine were excluded because of their high absorbance at 230 nm. Representative van Holde–Weischet distribution plots are shown in Figure 4, and the s_w data are

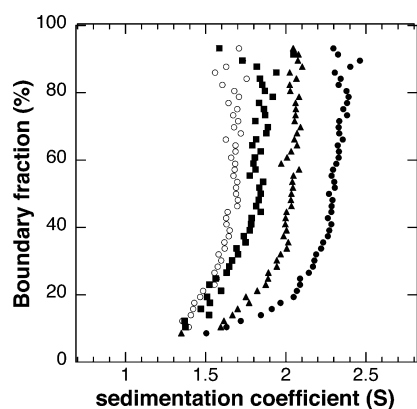


Figure 4. van Holde–Weischet distribution plot for RDPHeH $_{25-117}$ ($\sim 15 \mu\text{M}$ total monomer) without (○) and with 50 mM leucine (■), 50 mM norleucine (▲), or 1 mM L-phenylalanine (●).

summarized in Table 1. D-Phenylalanine (5 mM) and (S)-2-amino-3-phenyl-1-propanol (10 mM) yielded obvious increases in the s_w value of 15 μM RDPHeH $_{25-117}$. When the concentrations of the other compounds were increased to 50 mM, L-norleucine showed a clear increase in the s_w value, while the s_w values with L-leucine, L-isoleucine, and L-valine were not significantly different from the value in the absence of phenylalanine. However, direct inspection of the van Holde–Weischet plots shows that the changes with leucine (Figure 4) and isoleucine are significant. In contrast, no reproducible change in the s_w value was seen in the presence of valine (results not shown). None of the other compounds examined affected the s_w value of RDPHeH $_{25-117}$. These results are consistent with the NMR analyses.

Activation of PheH. Activation of PheH by phenylalanine is accompanied by a significant structural change resulting in an increase in the fluorescence emission of the protein.^{15,33} This change was used to identify compounds that activate PheH. The binding to intact PheH of the compounds that stabilized

the RDPHeH dimer was analyzed by fluorescence spectroscopy, with the exception of the highly fluorescent L-4-aminophenylalanine. L-Alanine and L-serine were also examined as representative amino acids that do not stabilize the RDPHeH dimer. Figure 5 shows the fluorescence change of 5 μM PheH as a function of the concentration of each compound. Also shown for comparison is the effect of L-phenylalanine. All of the molecules that stabilize the RDPHeH dimer caused a change in the fluorescence of PheH. A very small fluorescence change was seen in the presence of 50 mM valine; at only $\sim 2\%$ of the maximal fluorescence change seen with phenylalanine and methionine, this was at the limit of detection. The compounds that did not stabilize the RDPHeH dimer did not alter the fluorescence of the protein even at a concentration of 50 mM (results not shown).

PheH binding by phenylalanine is cooperative.^{12,15} The concentration dependences of the fluorescence changes in Figure 5 were all fit better by the Hill equation than by the equation for noncooperative binding, with an average Hill coefficient of 2.5 for L- and D-phenylalanine and for (S)-2-amino-3-phenyl-1-propanol and lower values for norleucine and methionine. The resulting fits are shown in Figure 5, and the dissociation constants (K_d) are listed in Table 1. All of the compounds examined bound 2–3 orders of magnitude more weakly than L-phenylalanine. For those compounds for which no fluorescence changes were observed even at 50 mM, only a lower limit of 500 mM for the K_d can be estimated.

Finally, the abilities of these compounds to activate PheH were examined directly in enzyme assays. PheH was preincubated with each compound at 10 or 50 mM (1 mM for L-phenylalanine) for 10 min before determining the activity with 1 mM phenylalanine as the substrate. The results are shown in Figure 6 and Table 1. The effects of the compounds on PheH activity are consistent with the effects on the fluorescence spectrum. L-Phenylalanine, D-phenylalanine, (S)-2-amino-3-phenyl-1-propanol, L-norleucine, and L-methionine all activated PheH ~ 7 -fold. Lower levels of activation were seen with L-4-aminophenylalanine, 3-phenylpropionate, L-leucine, and L-isoleucine; this is consistent with the very low affinities of these compounds. The activation by valine is barely significant, consistent with the small effects of this amino acid on fluorescence and dimerization.

Table 1. Effects of Amino Acids and Phenylalanine Analogues on RDPheH₂₅₋₁₁₇ and PheH

ligand	K_d value from fluorescence ^a (mM)	activation ^b	NMR spectral changes ^c	s_w (S) ^d
none		1.0		1.54 ± 0.03
L-phenylalanine	0.054 ± 0.003 (2.4 ± 0.3) ^e	6.0 ± 0.5	yes	2.11 ± 0.02
D-phenylalanine	8.4 ± 0.5 (2.7 ± 0.4)	7.1 ± 0.1	yes	1.76 ± 0.04
(S)-2-amino-3-phenyl-1-propanol	10.6 ± 2.0 (2.3 ± 0.8)	6.5 ± 0.1	yes	1.77 ± 0.01
L-4-aminophenylalanine	ND ^f	3.2 ± 0.3	yes	ND ^f
L-norleucine	17 ± 3 (1.6 ± 0.2)	6.6 ± 0.8	yes	1.80 ± 0.01
L-methionine	24 ± 4 (1.5 ± 0.2)	5.8 ± 1.4	yes	ND ^f
L-leucine	93 ± 3	2.2 ± 0.5	yes	1.58 ± 0.01
3-phenylpropionic acid	173 ± 5	1.8 ± 0.4	yes	ND ^f
L-isoleucine	333 ± 6	1.5 ± 0.1	yes	1.59 ± 0.03
L-valine	≥500	1.2 ± 0.1	– ^g	1.59 ± 0.01
L-alanine	>500	1.1 ± 0.1	no	1.51 ± 0.03
L-serine	>500	1.0 ± 0.1	no	1.54 ± 0.03

^aBased on the fluorescence change of 5 μ M PheH in the presence of each compound. ^bThe relative activation of PheH upon preincubation with the indicated compounds at a concentration of 50 mM [1 mM was used for L-phenylalanine and 10 mM for D-phenylalanine, (S)-2-amino-3-phenyl-1-propanol, and L-4-aminophenylalanine]. ^cChanges in the ¹H–¹⁵N HSQC NMR spectrum of 400 μ M RDPheH₂₅₋₁₁₇ consistent with dimerization in the presence of the indicated compound at 50 mM [1 mM for L-phenylalanine and 10 mM for D-phenylalanine, (S)-2-amino-3-phenyl-1-propanol, and L-4-aminophenylalanine]. ^dThe s_w values calculated by ~ 15 μ M RDPheH₂₅₋₁₁₇ mixed with each compound at 50 mM [1 mM for L-phenylalanine, 5 mM for D-phenylalanine, and 10 mM for (S)-2-amino-3-phenyl-1-propanol]. ^eHill coefficient. ^fNot determined. ^gOnly a partial shift to the dimer could be detected by NMR.

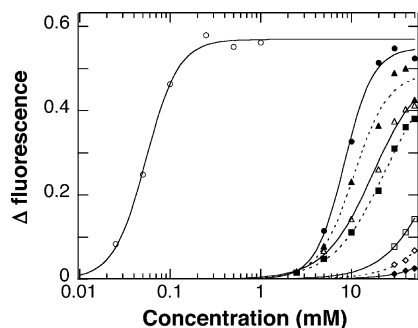


Figure 5. Fluorescence changes upon binding of selected amino acids and phenylalanine analogues to PheH (5 μ M) in 0.2 M HEPES (pH 7.5) at 25 °C: L-phenylalanine (○), D-phenylalanine (●), (S)-2-amino-3-phenyl-1-propanol (▲), L-norleucine (△), L-methionine (■), L-leucine (□), 3-phenylpropionic acid (◇), and L-isoleucine (◆). The lines are from fits of the data to $\Delta F_{\text{fluorescence}} = \Delta F_{\text{max}} \times [\text{aa}]^n / (K_d^n + [\text{aa}]^n)$. To fit the data for leucine, 3-phenylpropionic acid, and isoleucine, the maximal fluorescence change was fixed at the average value for the other compounds.

DISCUSSION

While studies of the isolated regulatory domains of PheH support a model in which activation of PheH involves formation of an RDPheH dimer, direct structural evidence of such a model in the context of intact PheH is lacking. If activation of PheH does involve dimerization of the regulatory domain, compounds that stabilize the regulatory domain dimer should activate PheH. The results of the experiments described here show that compounds that stabilize the RDPheH dimer do indeed activate the intact enzyme, providing support for regulatory domain dimerization being involved in the conformational change associated with activation.

The NMR spectra of Figures 1 and 2 are consistent with the RDPheH dimer being a symmetrical side-by-side dimer resembling the dimer formed by the ACT core of the regulatory domain of tyrosine hydroxylase.⁶ The lack of significant differences in the HSQC spectra of the

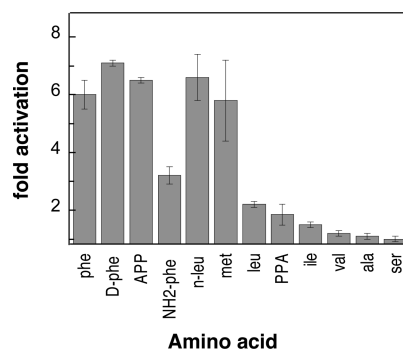


Figure 6. Activation of PheH by different amino acids and phenylalanine analogues. PheH (25 μ M) was incubated with the indicated compounds at 23 °C for 10 min before measuring the activity with phenylalanine as a substrate. The concentrations of potential activators during the preincubation were 1 mM for L-phenylalanine, 10 mM for D-phenylalanine, (S)-2-amino-3-phenyl-1-propanol (APP), and L-4-aminophenylalanine (NH₂-phe), and 50 mM for the others. PPA denotes 3-phenylpropionate and n-leu norleucine.

RDPheH₂₅₋₁₁₇ dimer formed at a high concentration in the absence of phenylalanine and that formed at a lower protein concentration in the presence of sufficient phenylalanine to form $\sim 100\%$ dimer establishes that the peptide backbone is not significantly perturbed in forming the dimer. This supports our previous proposal that phenylalanine binds after dimer formation²⁴ and suggests that the interactions with phenylalanine in the dimer involve primarily amino acid side chains. The dimer of the regulatory domain of tyrosine hydroxylase provides a structural model for the RDPheH dimer; this dimer is stabilized primarily by backbone interactions.⁶ A side-by-side RDPheH dimer is consistent with the structure proposed by Jaffe et al.²⁷ for activated PheH.

NMR spectroscopy and analytical ultracentrifugation were used as complementary probes for formation of the RDPheH dimer, although the two approaches require very different protein concentrations. The NMR spectra provide residue-specific information about the effects of dimerization, while centrifugation is a direct measure of the relative amounts of

dimer and monomer. The NMR spectra were collected at a high protein concentration, where only ~20% of the protein is monomeric. These conditions were such that even weakly binding compounds would shift all of the protein to dimer, eliminating the signals arising from the monomer. Centrifugation was conducted at a 26-fold lower protein concentration, at which the protein is ~70% monomer and the s_w value is most sensitive to an increased level of dimerization. The results with the most weakly activating compounds suggest that the NMR spectra provide a more sensitive probe for dimerization than AUC. Fluorescence spectroscopy and activity assays similarly provide complementary probes of activation. Critically, Phillips et al.¹⁵ showed that these two methods give quantitative agreement for the concentration dependence of activation by phenylalanine. The extent of activation by PheH that is measured in activity assays is sensitive to the conditions of the assay, in part because activation occurs during the assay, because it contains phenylalanine. In contrast, following activation by the change in protein fluorescence can be done without phenylalanine or BH₄ being present.

The probes of RDPheH dimerization and of activation of intact PheH agree on the structural requirements for activation, consistent with regulatory domain dimerization being involved in the conformational change associated with activation. The specificity for the amino acid side chain is quite high. Amino acids other than phenylalanine with hydrophobic side chains of a comparable size, such as methionine, leucine, and norleucine, have K_d values of 10–100 mM. β -Branching further decreases the affinity, based on the results with leucine and isoleucine. The effects of valine and isoleucine on activity and dimerization are at the limits of detection for all the methods used here, but for both amino acids, the effects on dimerization and activation are consistent with the K_d values measured by fluorescence. Tryptophan was not examined in the experiments described here because of its fluorescence and limited solubility, but Kaufman et al. previously reported that PheH is activated by 28 mM tryptophan.³⁶ Tyrosine was not examined for the same reasons. L-4-Aminophenylalanine can be considered a tyrosine analogue; the introduction of the amino moiety weakens binding by 2 orders of magnitude, suggesting that tyrosine also binds weakly to the allosteric site. The α -carboxylate appears to be worth 3–4 kcal/mol, based on the difference between L-phenylalanine and (S)-2-amino-3-phenyl-1-propanol. This is consistent with the loss of a favorable ionic interaction between the carboxylate and a positively charged amino acid side chain. D-Phenylalanine has an affinity comparable to that of (S)-2-amino-3-phenyl-1-propanol; loss of the interaction with the carboxylate of the former would explain this result. 3-Phenylpropionate binds 1 order of magnitude more weakly than D-phenylalanine and (S)-2-amino-3-phenyl-1-propanol, suggesting that the amino group of phenylalanine is more important for binding in the allosteric site than is the carboxylate. The changes in the NMR spectrum in the presence of either (S)-2-amino-3-phenyl-1-propanol or 3-propionate are slightly different from those seen with the activating amino acids, suggesting that the lack of the carboxylate or amino group may result in a slightly altered binding mode. These results are consistent with previous analyses of the ability of different amino acids to activate PheH, in that D-phenylalanine, L-norleucine, and L-methionine were previously reported to activate PheH at high concentrations.³⁶

It is unlikely that any of the amino acids in Table 1 other than phenylalanine are physiologically important activators of

PheH, because the concentrations in the liver of those amino acids that activate the enzyme are normally 50–100 μ M.^{37,38} However, besides the traditional low-phenylalanine dietary treatment for PKU, supplementation with large neutral amino acids (LNAAs) has been demonstrated to be a successful therapy to further reduce phenylalanine levels in the brain and blood.^{39–42} This effect has been attributed to competition of the LNAAs with phenylalanine for the L-amino acid carrier across the blood–brain barrier.⁴¹ These results raise the possibility that some LNAAs also activate PheH.

For all of the compounds with K_d values below their solubility limits, the binding to PheH measured by fluorescence was cooperative, with an average Hill coefficient of ~2. This cooperativity can be explained by the ability of the RDPheH dimer to bind one molecule of phenylalanine per monomer or two per dimer. Our previous analyses of the effects of phenylalanine on the quaternary structure of RDPheH supported a sequential model for allostery in which dimerization precedes phenylalanine binding and two phenylalanine molecules bind sequentially to the dimer.²⁴ This stoichiometry is also supported by our calorimetric studies of RDPheH_{25–117} (C. O. Khan, S. Zhang, and P. F. Fitzpatrick, unpublished observations).

Overall, the results described here support the proposal that activation of PheH by phenylalanine is linked to the dimerization of the regulatory domains. The results also provide additional evidence that the allosteric site for activation of PheH by phenylalanine is located in the regulatory domain.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00616.

Figure S1 (PDF)

■ 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 (NIH) Grant R01 GM098140 (to P.F.F.). The NMR Spectroscopy Core at the University of Texas Health Science Center is supported in part by NIH Grant NCI/CA054174 and Grant RP128067 from the Cancer Prevention and Research Institute in Texas (to A.P.H.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Support for the Center for Macromolecular Interactions and the Center for NMR Spectroscopy from the Office of the Vice President for Research at the University of Texas Health Science Center is gratefully acknowledged. We thank Virgil Schirf for assistance with the AUC experiments, Crystal Khan for assistance with the fluorescence measurements, and Dr. Kristin Cano-McCue for assistance with NMR spectroscopy.

■ ABBREVIATIONS

PheH, phenylalanine hydroxylase; RDPheH, regulatory domain of rat phenylalanine hydroxylase, residues 1–117; RDPheH_{25–117}, RDPheH lacking the first 24 residues; AUC, analytical ultracentrifugation; s_w , weight-average sedimentation coefficient; HSQC, heteronuclear single-quantum correlation; BH₄, tetrahydrobiopterin; LNAAs, large neutral amino acids.

■ REFERENCES

- (1) Fitzpatrick, P. F. (1999) Tetrahydropterin-dependent amino acid hydroxylases. *Annu. Rev. Biochem.* 68, 355–381.
- (2) 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.
- (3) Goodwill, K. E., Sabatier, C., Marks, C., Raag, R., Fitzpatrick, P. F., and Stevens, R. C. (1997) Crystal structure of tyrosine hydroxylase at 2.3 Å and its implications for inherited neurodegenerative diseases. *Nat. Struct. Biol.* 4, 578–585.
- (4) Wang, L., Erlandsen, H., Haavik, J., Knappskog, P. M., and Stevens, R. C. (2002) Three-dimensional structure of human tryptophan hydroxylase and its implications for the biosynthesis of the neurotransmitters serotonin and melatonin. *Biochemistry* 41, 12569–12574.
- (5) Fitzpatrick, P. F. (2003) Mechanism of aromatic amino acid hydroxylation. *Biochemistry* 42, 14083–14091.
- (6) Zhang, S., Huang, T., Ilangovan, U., Hinck, A. P., and Fitzpatrick, P. F. (2014) The solution structure of the regulatory domain of tyrosine hydroxylase. *J. Mol. Biol.* 426, 1483–1497.
- (7) 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.
- (8) Xia, T., Gray, D. W., and Shiman, R. (1994) Regulation of rat liver phenylalanine hydroxylase. III. Control of catalysis by (6R)-tetrahydrobiopterin and phenylalanine. *J. Biol. Chem.* 269, 24657–24665.
- (9) Fitzpatrick, P. F. (2012) Allosteric regulation of phenylalanine hydroxylase. *Arch. Biochem. Biophys.* 519, 194–201.
- (10) Haycock, J. W. (1990) Phosphorylation of tyrosine hydroxylase *in situ* at serine 8, 19, 31, and 40. *J. Biol. Chem.* 265, 11682–11691.
- (11) Daubner, S. C., Le, T., and Wang, S. (2011) Tyrosine hydroxylase and regulation of dopamine synthesis. *Arch. Biochem. Biophys.* 508, 1–12.
- (12) Flydal, M. I., and Martinez, A. (2013) Phenylalanine hydroxylase: Function, structure, and regulation. *IUBMB Life* 65, 341–349.
- (13) 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.
- (14) Shiman, R., Jones, S. H., and Gray, D. W. (1990) Mechanism of phenylalanine regulation of phenylalanine hydroxylase. *J. Biol. Chem.* 265, 11633–11642.
- (15) Phillips, R. S., Parniak, M. A., and Kaufman, S. (1984) Spectroscopic investigation of ligand interaction with hepatic phenylalanine hydroxylase: Evidence for a conformational change associated with activation. *Biochemistry* 23, 3836–3842.
- (16) Kowlessur, D., Yang, X.-J., and Kaufman, S. (1995) Further studies of the role of Ser-16 in the regulation of the activity of phenylalanine hydroxylase. *Proc. Natl. Acad. Sci. U. S. A.* 92, 4743–4747.
- (17) Døskeland, 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.
- (18) 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.
- (19) 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.
- (20) 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.
- (21) 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.
- (22) Shiman, R., and Gray, D. W. (1980) Substrate activation of phenylalanine hydroxylase. A kinetic characterization. *J. Biol. Chem.* 255, 4793–4800.
- (23) 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.
- (24) 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.
- (25) Li, J., Ilangovan, 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.
- (26) Roberts, K. M., Khan, C. A., Hinck, C. S., and Fitzpatrick, P. F. (2014) Activation of phenylalanine hydroxylase by phenylalanine does not require binding in the active site. *Biochemistry* 53, 7846–7853.
- (27) 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.
- (28) Lang, E. J. M., Cross, P. J., Mittelstädt, G., Jameson, G. B., and Parker, E. J. (2014) Allosteric ACTion: the varied ACT domains regulating enzymes of amino-acid metabolism. *Curr. Opin. Struct. Biol.* 29, 102–111.
- (29) Marley, J., Lu, M., and Bracken, C. (2001) A method for efficient isotopic labeling of recombinant proteins. *J. Biomol. NMR* 20, 71–75.
- (30) 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.
- (31) Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 6, 277–293.
- (32) Johnson, B. A. (2004) Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol. Biol.* 278, 313–352.
- (33) Demeler, B. (2005) UltraScan: A comprehensive data analysis software package for analytical ultracentrifugation experiments. In *Modern Analytical Ultracentrifugation: Techniques and Methods* (Scott, D. J., Harding, S. E., and Row, A. J., Eds.) pp 210–229, Royal Society of Chemistry, London.
- (34) Schuck, P. (2000) Size-Distribution Analysis of Macromolecules by Sedimentation Velocity Ultracentrifugation and Lamm Equation Modeling. *Biophys. J.* 78, 1606–1619.
- (35) 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.
- (36) Kaufman, S., and Mason, K. (1982) Specificity of amino acids as activators and substrates for phenylalanine hydroxylase. *J. Biol. Chem.* 257, 14667–14678.

(37) Stegink, L. D., Filer, L. J., Brummel, M. C., Baker, G. L., Krause, W. L., Bell, E. F., and Ziegler, E. E. (1991) Plasma amino acid concentrations and amino acid ratios in normal adults and adults heterozygous for phenylketonuria ingesting a hamburger and milk shake meal. *Am. J. Clin. Nutr.* 53, 670–675.

(38) Ryan, W. L., and Carver, M. J. (1966) Free amino acids of human foetal and adult liver. *Nature* 212, 292–293.

(39) Williams, R. A., Mamotte, C. D. S., and Burnett, J. R. (2008) Phenylketonuria: An inborn error of phenylalanine metabolism. *Clin. Biochem. Rev.* 29, 31–41.

(40) van Spronsen, F. J., de Groot, M. J., Hoeksma, M., Reijngoud, D. J., and van Rijn, M. (2010) Large neutral amino acids in the treatment of PKU: from theory to practice. *J. Inherited Metab. Dis.* 33, 671–676.

(41) Pietz, J., Kreis, R., Rupp, A., Mayatepek, E., Rating, D., Boesch, C., and Bremer, H. J. (1999) Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria. *J. Clin. Invest.* 103, 1169–1178.

(42) Matalon, R., Michals-Matalon, K., Bhatia, G., Grechanina, E., Novikov, P., McDonald, J. D., Grady, J., Tyring, S. K., and Guttler, F. (2006) Large neutral amino acids in the treatment of phenylketonuria (PKU). *J. Inherited Metab. Dis.* 29, 732–738.