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

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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 diaminobiopterin-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,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.
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.27,28 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.29 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 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 (RDPheH25–117) were performed as previously described.24,25 For 15N-labeled RDPheH and RDPheH25–117 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.26,28 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 RDPheH25–117 precipitate too rapidly at pH <8 for NMR analyses, consistent with a calculated pI value of 6.8. NMR screening for formation of the RDPheH25–117 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/15C/15N) Micro-CryoProbe. NMR samples for screening were made using a Gilson 215 Liquid Handler. 15N-labeled RDPheH25–117 (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 SampleJet tube. All spectra were processed using NMRPipe31 and analyzed using NMRView.12

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 RDPheH25–117 (~15 μM total monomer) with detection at 230 nm. AUC samples were prepared in 50 mM phosphate and 100 mM NaCl (pH 8.0). Unispec III13 was used for van Holde–Weischat 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. 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 (RDPheH25–117) was more stable than full-length RDPheH, with a lower propensity to precipitate at NMR
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−15N HSQC spectrum of 3 mM RDPheH25−117 at pH 8.0. On the basis of the previously 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 RDPheH25−117. (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 RDPheH25−117 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 RDPheH25−117 (~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 RDPheH25−117, consistent with our previous model for phenylalanine binding.24 The HSQC spectrum of 3 mM RDPheH25−117 and that of 430 μM RDPheH25−117 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 RDPheH25−117 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 RDPheH25−117 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 RDPheH25−117 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
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 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.

**Figure 4.** van Holde–Weischet distribution plot for RDPheH$_{25-117}$ (∼15 μM total monomer) without (O) and with 50 mM leucine (●), 50 mM norleucine (▲), or 1 mM L-phenylalanine (■).

**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. 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 3.** Effects of selected amino acids on the 2D $^1$H–$^{15}$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$_2$O (pH 8.0), at 300 K at a magnetic field strength of 11.7 T (500 MHz for $^1$H).

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 5 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.

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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. The lack of significant differences in the HSQC spectra of the RDPheH$_{25-117}$ 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 ~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 formation 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 provides a structural model for the RDPheH dimer; this dimer.

Table 1. Effects of Amino Acids and Phenylalanine Analogues on RDPheH$_{25-117}$ and PheH

| ligand                  | $K_v$ value from fluorescence$^a$ (mM) | activation$^b$ | NMR spectral changes$^c$ | $s_e$ (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-amino-phenylalanine | 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      | ND$^f$                   |              |
| L-leucine               | 93 ± 3                                 | 2.2 ± 0.5      | yes                      | 1.58 ± 0.01  |
| 3-phenylpropionic acid  | 173 ± 5                                | 1.8 ± 0.4      | 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  |

$^a$Based on the fluorescence change of 5 μM PheH in the presence of each compound. $^b$The 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 L-phenylalanine, (S)-2-amino-3-phenyl-1-propanol, and L-4-aminophenylalanine]. $^c$Changes in the [H]-15N HSQC NMR spectrum of 400 μM RDPheH$_{25-117}$ consistent with dimerization in the presence of the indicated compound at 50 mM [1 mM for L-phenylalanine and 10 mM for L-phenylalanine, (S)-2-amino-3-phenyl-1-propanol, and L-4-aminophenylalanine]. $^d$The $s_e$ values calculated by $\sim$15 μM RDPheH$_{25-117}$ mixed with each compound at 50 mM [1 mM for L-phenylalanine, 5 mM for L-phenylalanine, and 10 mM for (S)-2-amino-3-phenyl-1-propanol]. $^e$Hill coefficient. $^f$Not determined. $^g$Only 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 μM) 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 fits of the data to $\Delta$fluorescence = $\Delta$Flmax × [aa]$^n$/([aa]$^n$ + [aa]$.5$). 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.

Figure 6. Activation of PheH by different amino acids and phenylalanine analogues. PheH (25 μM) was incubated with the indicated compounds at 25°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 L-phenylalanine, (S)-2-amino-3-phenyl-1-propanol, and L-4-aminophenylalanine. RDPheH$_{25-117}$ 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 ~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 formation 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. A side-by-side RDPheH dimer is consistent with the structure proposed by Jaffe et al. 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.
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 $K_d$ 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. 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 BH$_4$ 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. Tyrosine was not examined for the same reasons. L-4-Aminophenylanine 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.

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 μM. 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. This effect has been attributed to competition of the LNAAs with phenylalanine for the L-amino acid carrier across the blood–brain barrier. 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. 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)**

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**Notes**

The authors declare no competing financial interest.

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ABBREVIATIONS

PheH, phenylalanine hydroxylase; RDPheH, regulatory domain of rat phenylalanine hydroxylase, residues 1–117; RDPheH_{15–117}, RDPheH lacking the first 24 residues; AUC, analytical ultracentrifugation; \( s_w \), weight-average sedimentation coefficient; HSQC, heteronuclear single-quantum correlation; BH_{4}, tetrahydrobiopterin; LNAAs, large neutral amino acids.

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