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Direct Evidence for a Phenylalanine Site in the Regulatory Domain of Phenylalanine Hydroxylase

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Abstract

The hydroxylation of phenylalanine to tyrosine by the liver enzyme phenylalanine hydroxylase is regulated by the level of phenylalanine. Whether there is a distinct allosteric binding site for phenylalanine outside of the active site has been unclear. The enzyme contains an N-terminal regulatory domain that extends through Thr117. The regulatory domain of rat phenylalanine hydroxylase was expressed in *E. coli*. The purified protein behaves as a dimer on a gel filtration column. In the presence of phenylalanine, the protein elutes earlier from the column, consistent with a conformational change in the presence of the amino acid. No change in elution is seen in the presence of the non-activating amino acid proline. ¹H-¹⁵N HSQC NMR spectra were obtained of the ¹⁵N-labeled protein alone and in the presence of phenylalanine or proline. A subset of the peaks in the spectrum exhibits chemical shift perturbation in the presence of phenylalanine, consistent with binding of phenylalanine at a specific site. No change in the NMR spectrum is seen in the presence of proline. These results establish that the regulatory domain of phenylalanine hydroxylase can bind phenylalanine, consistent with the presence of an allosteric site for the amino acid.

The physiological role of the liver enzyme phenylalanine hydroxylase (PheH)1 is to catalyze the hydroxylation of excess phenylalanine in the diet to form tyrosine, using tetrahydrobiopterin (BH₄) as the source of electrons for this monooxygenation reaction (Figure 1) [1]. The importance of the enzyme is demonstrated by the devastating effects of insufficient PheH activity. The resulting disease, phenylketonuria, results in poor growth

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¹Abbreviations: PheH, phenylalanine hydroxylase; PheH₁₁₇, the regulatory domain of rat phenylalanine hydroxylase, lacking residues after Thr117; hisPheH₁₁₇, PheH₁₁₇ with an N-terminal histidine tag; TyrH, tyrosine hydroxylase; TrpH, tryptophan hydroxylase; BH₄, tetrahydrobiopterin; 6-MePH₄, 6-methyltetrahydropterin; PMSF, phenylmethylsulfonyl fluoride; HSQC, heteronuclear single-quantum coherence.

and progressive intellectual impairment, with eventual death of the affected patient at a young age in the absence of treatment [2].

PheH is a member of the family of aromatic amino acid hydroxylases, along with tyrosine hydroxylase (TyrH) and tryptophan hydroxylase (TrpH) [1]. TyrH hydroxylase in the adrenal gland and central nervous system catalyzes the rate-limiting step in the formation of catecholamine neurotransmitters. TrpH in the central nervous system catalyzes the rate-limiting step in the formation of serotonin. All three enzymes are homotetramers, with each monomer containing an N-terminal regulatory domain of 100-160 residues, a homologous catalytic domain of ~300 residues, and a C-terminal tetramerization domain of ~45 residues that contains a C-terminal helix. There is no structure available of an intact eukaryotic aromatic amino acid hydroxylase. In the case of PheH, the most complete structures are of a dimeric rat enzyme that lacks the C-terminal helix [3] and of the tetrameric human enzyme lacking the N-terminal regulatory domain [4]. The structure of the dimeric rat enzyme shows that the N-terminus of the regulatory domain extends across the active site opening. This feature has led to residues 19-29 being designated an auto-inhibitory domain critical for regulation of PheH [5].

Maintaining proper levels of PheH activity is clearly critical, and the enzyme activity is regulated in response to levels of free phenylalanine. The seminal work leading to our present understanding of the regulation of PheH was carried out by Shiman and coworkers [6-9]. PheH exhibits positive cooperativity when the rate of the reaction is determined as a function of phenylalanine concentration and hyperbolic kinetics when the concentration of the tetrahydropterin is varied. In addition, there is a lag in the formation of tyrosine unless the enzyme is first incubated with phenylalanine [9], and the lag is more pronounced if the enzyme is first incubated with a tetrahydropterin. In the model developed to explain these observations, the resting form of the enzyme is inactive. Binding of BH₄ stabilizes the inactive form, while binding of phenylalanine at a regulatory site activates the enzyme. The structure of the combined catalytic and regulatory domains of PheH provided a structural rationale for this model, by showing that residues 19-29 of the regulatory domain lie across the active site in the resting enzyme and presumably keep the enzyme from binding substrates [3]. Binding of phenylalanine at a regulatory site would then shift the enzyme to a conformation in which the active site was open. Direct structural evidence for this model is lacking. There is no reported structure of a form of PheH with both a regulatory domain and either phenylalanine or a pterin bound, so that the actual structural change that results in activation has not been established. In the absence of a direct structural support for a regulatory binding site for phenylalanine, the presence of such a site has come under question. A protein containing maltose binding protein fused to the N-terminus of the human PheH regulatory domain has been reported to bind radio-labeled phenylalanine [10]. In contrast, Thorolfsson et al. [11] could not detect any binding to the isolated regulatory domain by differential scanning calorimetry and concluded that activation is due to interactions between the active sites and that the role of the regulatory domain is to allow communication between active sites. Such a model would be consistent with the structure, which shows each regulatory domain interacting with two catalytic domains.

To address the existence of a regulatory binding site for phenylalanine in PheH, we have expressed the isolated regulatory domain of rat PheH and analyzed the effects of phenylalanine on its structure. The results establish that there is a binding site for phenylalanine in this domain, consistent with the existence of an allosteric site for phenylalanine in PheH.

Materials and Methods

Materials

Leupeptin and pepstatin A were from Peptides Institute, Inc. (Osaka, Japan). Restriction and DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA). $^{15}\text{NH}_4\text{Cl}$ was from Cambridge Isotope Laboratories, Inc (Andover, MA). Thiamine hydrochloride, imidazole, carbonic anhydrase, cytochrome c and thrombin were from Sigma-Aldrich (St. Louis, MO). Biotin and hemoglobin were from USB (Cleveland, Ohio).

Molecular biology

To construct a plasmid coding for the regulatory domain of rat PheH, Phe₁₁₇, a unique *NcoI* site was introduced into pERPH5, the expression plasmid for wild-type rat phenylalanine hydroxylase [12] to stop translation before residue 118. Site-directed mutagenesis was carried out using the oligonucleotide 5'-aag gaa aag aac aca tga CCA TGG ttc ccg cgg acc-3' with the Stratagene QuikChange Kit using *Pfu* DNA polymerase. (The mutated codons are indicated by upper case letters.) QIAfilter plasmid midi prep kits and QIAprep Spin miniprep kits were used to purify the resulting plasmids, which were used to transfect OmniMax competent cells (Invitrogen, Carlsbad, CA). Oligonucleotide synthesis and DNA sequencing were conducted at the Nucleic Acids Core Facility at the University of Texas Health Science Center. Once the mutation was confirmed, the DNA coding for the regulatory domain of rat PheH was moved to pET21b from pERPH5 by PCR. The oligonucleotide 5'-gg gaa ttc CAT ATG gca gct gtt gtc ctg gag aat gga-3' was used as the 5' primer to create a new *NdeI* site. The oligonucleotide 5'-ccg CTC GAG tca tgt gtt ctt ttc ctt tgc tcg-3' was used as the 3' primer to create a new *XhoI* site. After purification, the fragment encoding the regulatory domain gene was ligated to pET21b that had been treated with *NdeI* and *XhoI*. One positive clone was sequenced to confirm that the cDNA for the regulatory domain of phenylalanine hydroxylase was inserted between the *NdeI* and *XhoI* sites of pET21b. This plasmid was designated pETRD. Similar procedures were used to introduce the regulatory domain DNA into pET28a to produce a recombinant protein of the regulatory domain of PheH with a 6-histidine tag at the N-terminus, hisPheH₁₁₇. This plasmid was designated pEThisRD.

Expression and purification of the regulatory domain of PheH

E. coli strain C41(DE3) transformed with the plasmid pETRD was grown overnight at 37°C in Luria-Bertani medium plus 100 µg/mL ampicillin. Expression was induced by addition of 0.25 mM isopropyl β-D-thiogalactopyranoside when the A₆₀₀ reached 0.8–1.0. After 12–15 h, cells were harvested by centrifugation at 6000 × g for 30 min. Cell pellets were suspended in 50 mM Hepes, 0.2 M NaCl, 1 µM leupeptin, 1 µM pepstatin A, 100 µg/ml lysozyme, and 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), pH 7.5. Cells were lysed by sonication and the resulting cell suspension was centrifuged at 30,000 × g for 30 min. Solid ammonium sulfate was added to the supernatant, and protein precipitating between 60 and 80% saturation was collected and dissolved in 50 mM Hepes, 0.5 mM EDTA, 10% glycerol, 1 µM leupeptin, and 1 µM pepstatin A, pH 7.5. After dialysis against the same buffer, the protein was applied to a 2.5 × 14 cm column of Q-Sepharose equilibrated with the same buffer. The column was washed with 150 ml of the same buffer, and the protein was eluted with a 500 ml gradient of the buffer containing 0–0.2 M NaCl. The fractions were assayed by SDS-polyacrylamide gel electrophoresis. Those fractions showing a band with an apparent molecular weight of 13,000 were pooled and concentrated using an Amicon Ultra centrifugal filter (10,000 molecular weight cutoff, Millipore). The concentrated sample was then applied to a HiPrep 16/60 Sephacryl S-100 HR (GE Healthcare life science, Piscataway, NJ) gel filtration column in 50 mM Hepes, 0.2 M NaCl, 0.5 mM EDTA, 1 µM leupeptin and 1 µM pepstatin A, pH 7.5. The fractions were assayed by SDS-polyacrylamide

gel electrophoresis. Those exhibiting a single band with an apparent molecular weight of 13,000 were pooled and stored at -80°C . The yield from 1 liter of cell culture was 3–5 mg. The concentration of the purified protein was determined using an ϵ_{280} value of $8.94\text{ mM}^{-1}\text{cm}^{-1}$, calculated by the method of Pace et al. [13]. The single band on an SDS-polyacrylamide gel of purified PheH₁₁₇ was sent to the Institutional Mass Spectrometry Laboratory of the University of Texas Health Science Center for identification. Twelve unique peptides generated by trypsin digestion covering 114 of the 117 residues could be detected, confirming the protein as PheH₁₁₇.

To prepare ^{15}N -labeled protein for NMR, the plasmid pEThisRD was transformed into *E. coli* strain BL21(DE3). One liter of auto-inducing minimal medium used for expression was made from 1 g $^{15}\text{NH}_4\text{Cl}$, 50 mg kanamycin, 50 mM Na_2HPO_4 , 50 mM KH_2PO_4 , 5 mM Na_2SO_4 , 2 mM MgSO_4 , 0.5% glycerol, 0.05% glucose, 0.2% lactose and 0.5x trace metals, pH 7.5 [14]. Yeast extract (0.02%, w/v), 1 $\mu\text{g/ml}$ thiamine hydrochloride and 1 $\mu\text{g/ml}$ biotin were added to improve expression. All the components were autoclaved for sterilization, except that thiamine hydrochloride, biotin, trace metals and kanamycin were sterilized using a 0.22 μm syringe filter (Millipore, Bedford, MA). A freshly transformed single colony was inoculated into 100 ml auto-inducing minimal medium; the culture was left shaking at 300 rpm overnight at 37°C . This culture was then diluted into 900 ml of the same medium and kept shaking at 37°C for 5–8 h reached saturation (about 2.6–2.8). Then the temperature was adjusted to 25°C to until the A_{600} express the target protein. After 18–24 h, the cells were harvested by centrifugation at $6000 \times g$ for 30 min. Cell pellets were suspended in 20 mM sodium phosphate buffer, 0.5 M NaCl, 5 mM imidazole, 1 μM leupeptin, 1 μM pepstatin A, 100 $\mu\text{g/ml}$ lysozyme, and 100 $\mu\text{g/ml}$ PMSF, pH 7.5. Cells were lysed by sonication and the resulting suspension was centrifuged at $30,000 \times g$ for 30 min. The supernatant was applied to a HiTrap FF column (5 ml, GE Healthcare life science, Piscataway, NJ). A linear gradient of 50–300 mM imidazole in 10 column volumes of 20 mM sodium phosphate buffer, 0.5 M NaCl, pH 7.5, was used to elute the protein after first washing the column with 5 column volumes of 50 mM imidazole in the same buffer. The absorbance of the eluted fractions was monitored continuously at 280 nm. The peak fractions were pooled and concentrated using an Amicon Ultra centrifugal filter. The concentrated protein was dialyzed against 20 mM sodium phosphate buffer, 5% glycerol, 1 μM leupeptin, 1 μM pepstatin A, pH 7.5. To remove the histidine tag, thrombin (0.2 unit/ μl) was added to a solution of hisPheH₁₁₇ at approximate 1 mg/ml to yield a ratio of 4 units of thrombin per mg hisPheH₁₁₇. The mixture was gently shaken overnight at 4°C . The cleaved protein was purified using a HiPrep 16/60 Sephacryl S-100 HR gel filtration column as described for PheH₁₁₇. The yield of hisPheH₁₁₇ was 5–8 mg from 1 liter of cell culture. An SDS-polyacrylamide gel showed that thrombin completely removed the histidine tag.

Size exclusion chromatography

The molecular weight of PheH₁₁₇ was determined using a HiPrep 16/60 Sephacryl S-100 HR gel filtration column in 50 mM Hepes, 0.2 M NaCl, 0.5 mM EDTA, 1 μM leupeptin and 1 μM pepstatin A, pH 7.5, at 23°C . The flow rate was 0.8 ml/min and the absorbance of the eluate was monitored continuously at 280 nm. The standards used were hemoglobin (66,000), carbonic anhydrase (29,000) and cytochrome c (12,500). To determine the effects of proline or phenylalanine, the enzyme was incubated in buffer containing the amino acid at a concentration of 5 mM for at least 10 minutes at 23°C before loading the sample onto the column. The running buffer also contained the amino acid at a concentration of 5 mM.

NMR Spectroscopy and data processing

Samples for NMR containing 0.3 mM ^{15}N -labelled PheH₁₁₇ were prepared in 20 mM sodium phosphate buffer, 5% glycerol, pH 7, in 95% H_2O /5% D_2O . All NMR data were

collected at 308 K on a Bruker 700 MHz spectrometer using a cryogenically-cooled 5 mm probe equipped with ^{13}C and ^{15}N decouplers and pulsed field gradient coils. The ^1H - ^{15}N heteronuclear single-quantum coherence (HSQC) NMR spectrum [15] was recorded without any ligands and with 5 mM phenylalanine or 5 mM proline. NMR data were processed using NMRpipe and visualized using NMRDraw [16].

Results

Expression of the regulatory domain of rat PheH

Selection of the C-terminal end of the regulatory domain of rat PheH was based on analyses of available structures of PheH and of sequences of all three aromatic amino acid hydroxylases. The first structure to be determined of human PheH was of a protein lacking residues 1-102 and the C-terminal 25 residues [17]; residues 103-116 are not seen in that structure, suggesting they are disordered. (The numbering for the human and rat enzymes is the same.) A subsequent structure was determined of a protein lacking only the N-terminal 116 residues; in this case residue 117 is included in the structure [4]. Comparison of the sequences of all three aromatic amino acid hydroxylases from rat and human (Figure 2) shows that Val118 and subsequent residues in PheH are conserved in all three enzymes, suggesting that they are part of the homologous catalytic domains of these enzymes. All three enzymes retain full catalytic activity when the residues preceding Val118 are deleted [12,18–20]. The X-ray structures of the catalytic domains of TyrH and TrpH similarly begin with the residue preceding this valine [21,22], although the protein used for crystallization of TyrH contained additional residues at the N-terminus. Consequently, the codon for Val118 was replaced with a stop codon in the expression plasmid to yield a protein terminating at Thr117, PheH₁₁₇. PheH₁₁₇ expresses well in *E. coli*, reaching approximately 5% of the total protein, and is soluble, suggesting that it is properly folded. The protein is readily purified using ion exchange and size exclusion chromatography (Figure 3). Its identity as PheH₁₁₇ was confirmed by mass spectrometry of a tryptic digest.

Size exclusion chromatography

The quaternary structure of PheH₁₁₇ was analyzed using size exclusion chromatography. The protein elutes as a single peak with a retention time consistent with a molecular mass of 26,000. Since the sequence of the protein predicts a molecular mass for the polypeptide of 13,200, this result suggests that the regulatory domain of PheH is a dimer in solution. To determine if phenylalanine can bind to the isolated regulatory domain, PheH₁₁₇ was incubated with phenylalanine and then loaded on the size exclusion column, with phenylalanine included in the running buffer. Under these conditions the protein elutes from the column significantly earlier than in the absence of phenylalanine (Figure 4). This result is consistent with phenylalanine binding to regulatory domain of PheH, leading to a change to a more extended conformation. Recent analyses by H/D exchange mass spectrometry of the conformational change that occurs in PheH when phenylalanine binds are also consistent with phenylalanine binding leading to a more open structure [23]. As a control, a similar size exclusion analysis was done with proline, since proline has been reported not to activate PheH [24]. In contrast to the result with phenylalanine, in the presence of proline the retention time is unchanged from that of the protein alone (Figure 4).

NMR Spectroscopy

As an alternative approach, NMR spectroscopy was used to analyze the effects of phenylalanine on the structure of PheH₁₁₇. A histidine-tagged form of PheH₁₁₇ was expressed in minimal media containing $^{15}\text{NH}_4\text{Cl}$; the histidine-tag was then removed by proteolysis to yield ^{15}N -labeled PheH₁₁₇. This protein is soluble at concentrations up to 0.3 mM when the pH is kept above 7 (results not shown). The ^1H - ^{15}N HSQC NMR spectrum of

PheH₁₁₇ at pH 7.0 and 308 K is shown in Figure 5A. The spectrum shows reasonable dispersion, consistent with a folded protein.

Chemical shift perturbation was used to monitor the effect of phenylalanine on PheH₁₁₇. In the absence of phenylalanine, about 120 peaks can be seen in the spectrum. A number of cross peaks are broad and weak. After the addition of 5 mM phenylalanine, fewer peaks are seen in the spectrum and the intensities of the remaining peaks are greater and more uniform (Figure 5B). These changes provide direct evidence for binding of phenylalanine to the regulatory domain of PheH resulting in a conformational change. In contrast to the effect of phenylalanine, the presence of 5 mM proline has no effect on the NMR spectrum (Figure 5C). Direct comparison of the spectra in the absence and presence of phenylalanine (Figure 5D) illustrates that only a subset of the resonances are altered in the presence of amino acid, consistent with binding at a specific site.

Discussion

Phenylalanine is both a substrate and effector of PheH. While the model developed by Shiman and coworkers to describe the activation of the enzyme by phenylalanine proposes a second binding site for phenylalanine outside the active site [9], direct structural evidence for such a site is lacking and the existence of such a site has been questioned [11,25]. The data presented here provide clear evidence for a binding site for phenylalanine in the isolated regulatory domain of PheH. This result establishes that there is a binding site for the amino acid substrate separate from the active site, consistent with the existence of the proposed regulatory site. In support of this conclusion, both the gel filtration and the NMR analyses show that phenylalanine but not proline causes a conformational change in the isolated regulatory domain, as expected for binding of an effector at an allosteric site. The gel filtration data support the conclusion from the earlier analysis of H/D exchange by mass spectrometry that binding of phenylalanine to the regulatory domain of wild-type PheH leads to a conformational change that exposes the interface between the regulatory and catalytic domains [23].

The HSQC NMR spectrum of PheH₁₁₇ establishes that the isolated regulatory domain is well-folded in solution. The NMR spectrum in the absence of phenylalanine suggests that the regulatory domain has a dynamic structure in the absence of an activating amino acid, in that a number of peaks in the spectrum exhibit exchange broadening. The changes in the presence of phenylalanine are consistent with a shift to a more homogeneous conformation. This is in agreement with the regulatory model in which PheH exists as an inactive form in the absence of phenylalanine, shifting to the active form in its presence. For such a model, the active and inactive conformations must be in a dynamic equilibrium in the absence of the amino acid, with phenylalanine binding shifting the equilibrium to the active conformation. The effect of phenylalanine on the behavior of PheH₁₁₇ on a size exclusion column suggests that the active conformation is either more open or less symmetrical than the inactive conformation.

In their description of the three-dimensional structure of the combined catalytic and regulatory domains of PheH, Kobe et al. [3] noted the similarity of the structure of the regulatory domain to the regulatory domain of phosphoglycerate dehydrogenase. That enzyme is allosterically inhibited by binding of serine to a regulatory domain. Similar amino-acid regulatory domains have been noted in a number of allosteric proteins and have been termed ACT domains [26]. ACT domains exhibit variety in their arrangements within the larger protein structure. While the regulatory domains of PheH do not appear to interact with one another directly in the native protein, the more common arrangement is for two or even three domains to associate. The data of Figure 3 establish that the isolated PheH

regulatory domain forms dimers in solution. A reasonable explanation for this is that the residues that normally form the interface between the catalytic and regulatory domains of the enzyme form a new dimeric interface to avoid exposure to solvent.

The regulatory binding sites for amino acids in ACT domains do not appear to be strictly conserved. Rather, the ligands bind at or near domain interfaces, altering their relative orientations [26]. We recently used solvent H/D exchange as monitored by mass spectrometry to probe the conformational changes that occur in PheH upon phenylalanine binding [23]. In enzyme lacking the regulatory domain, phenylalanine only altered the exchange of residues involved in binding the amino acid substrate in the active site. Phenylalanine binding had a much greater effect on the intact protein, increasing the exchange of multiple peptides at the interface of the regulatory domain, consistent with a conformational change upon phenylalanine binding that moved the N-terminus away from the active site by altering the relative orientation of the two domains. In addition, phenylalanine binding altered the H/D exchange kinetics of residues in the regulatory domain outside of the interface between the two domains, supporting the existence of a distinct binding site for phenylalanine in the regulatory domain.

In conclusion, the results described here provide evidence for a binding site for phenylalanine in the regulatory domain of PheH. Binding of phenylalanine, but not the non-activating amino acid proline, at that site alters the conformation of the regulatory domain, as expected for an allosteric site involved in regulation.

Research Highlights

The regulatory domain of rat phenylalanine hydroxylase was expressed in bacteria.
Gel filtration and NMR spectroscopy show that the purified domain binds phenylalanine.

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Figure 1.
The reaction catalyzed by phenylalanine hydroxylase

Rat PheH	TVHELSDKE	KNT <u>VPWF</u> PRT	IQ <u>ELDR</u> FAN
Human PheH	TVHELSDKK	KDT <u>VPWF</u> PRT	IQ <u>ELDR</u> FAN
Rat TrpH	PDNIWTEEEE	LED <u>VPWF</u> PRK	ISE <u>ELDR</u> CSH
Human TrpH	PENIWTEEEE	LED <u>VPWF</u> PRK	ISE <u>ELDK</u> CSH
Human TyrH	QVSEDVRSPA	GPK <u>VPWF</u> PRK	VSE <u>ELDK</u> CHH
Rat TyrH	RVSDDVRSAR	EDK <u>VPWF</u> PRK	VSE <u>ELDK</u> CHH

Figure 2.

Sequence alignment of the aromatic amino acid hydroxylases at the junction between the regulatory and catalytic domains. Residues conserved in all three enzymes are in bold. Val118 in PheH and the corresponding residues in the other proteins are underlined.

Figure 3. SDS-Polyacrylamide gel electrophoresis of samples obtained during purification of PheH₁₁₇. The gel contained 15% (w/v) polyacrylamide. From left to right: sample after precipitating between 60 and 80% saturated ammonium sulfate, eluate from the Q-Sepharose column, eluate from the gel filtration column.

Figure 4. Size exclusion chromatography of the regulatory domain of PheH alone (circles) or in the presence of 5 mM phenylalanine (triangles) or proline (squares).

Figure 5. ^{15}N HSQC NMR spectra of the ^{15}N -labeled PheH₁₁₇ alone (A), in the presence of 5 mM phenylalanine (B) or in the presence of 5 mM proline (C). Panel D shows an overlay of the spectra in the absence (black) and presence (red) of 5 mM phenylalanine.