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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 *Escherichia 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. $^{1}H–^{15}N$ HSQC NMR spectra were obtained of the ^{15}N-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.

Introduction

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 (BH_4) as the source of electrons for this monooxygenation reaction (Fig. 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 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 autoinhibitory 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

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[1] *Abbreviations used*: PheH, phenylalanine hydroxylase; $PheH_{117}$, the regulatory domain of rat phenylalanine hydroxylase, lacking residues after Thr_{117}; $hisPheH_{117}$, $PheH_{117}$ with an N-terminal histidine tag; TyrH, tyrosine hydroxylase; TrpH, tryptophan hydroxylase; BH_4, tetrahydrobiopterin; 6-$MePH_4$, 6-methyltetrahydropterin; PMSF, phenylmethylsulfonyl fluoride; HSQC, heteronuclear single-quantum coherence.

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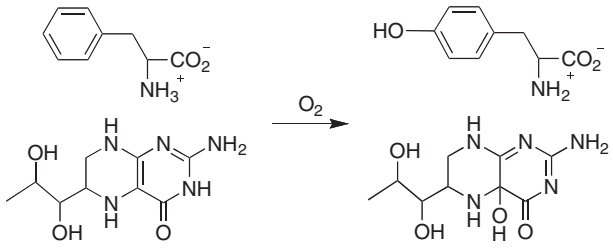

Fig. 1. The reaction catalyzed by phenylalanine hydroxylase.

with a tetrahydropterin. In the model developed to explain these observations, the resting form of the enzyme is inactive. Binding of BH_4 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). $^{15}NH_4Cl$ 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, Phe_{117}, 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 gtc 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, $hisPheH_{117}$. This plasmid was designated pEThisRD.

Expression and purification of the regulatory domain of PheH

Escherichia 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-thioglucanopyranoside when the A_{600} 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 phenylmethylsufonyl 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 l of cell culture was 3–5 mg. The concentration of the purified protein was determined using an e_{280} value of 8.94 $mM^{-1}cm^{-1}$, calculated by the method of Pace et al. [13]. The single band on an SDS–polyacrylamide gel of purified $PheH_{117}$ 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 $PheH_{117}$.

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 $^{15}NH_4Cl$, 50 mg kanamycin, 50 mM Na_2HPO_4, 50 mM KH_2PO_4,