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Regulation of Phenylalanine Hydroxylase: Conformational Changes Upon Phenylalanine Binding Detected by H/D Exchange and Mass Spectrometry[†]

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Abstract

Phenylalanine acts as an allosteric activator of the tetrahydropterin-dependent enzyme phenylalanine hydroxylase. Hydrogen/deuterium exchange monitored by mass spectrometry has been used to gain insight into local conformational changes accompanying activation of rat phenylalanine hydroxylase by phenylalanine. Peptides in the regulatory and catalytic domains that lie in the interface between these two domains show large increases in the extent of deuterium incorporation from solvent in the presence of phenylalanine. In contrast, the effects of phenylalanine on the exchange kinetics of a mutant enzyme lacking the regulatory domain are limited to peptides surrounding the binding site for the amino acid substrate. These results support a model in which the N-terminus of the protein acts as an inhibitory peptide, with phenylalanine binding causing a conformational change in the regulatory domain that alters the interaction between the catalytic and regulatory domains.

Phenylalanine hydroxylase (PheH)¹ is a mononuclear non-heme iron containing monooxygenase that catalyzes the hydroxylation of phenylalanine to tyrosine using molecular oxygen and tetrahydrobiopterin (BH₄) (1). This is the rate-limiting step in the catabolism of dietary phenylalanine in the liver. A deficiency in PheH causes the genetic disease phenylketonuria, a common metabolic disorder resulting in mental retardation (2). PheH belongs to the aromatic amino acid hydroxylase family, along with tyrosine hydroxylase (TyrH) and tryptophan hydroxylase (3). The latter two enzymes hydroxylate tyrosine and tryptophan in the biosynthetic pathways for the catecholamine neurotransmitters and serotonin, respectively. The three enzymes have similar chemical

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¹Abbreviations used: PheH, phenylalanine hydroxylase; Δ117PheH, rat PheH lacking the N-terminal 117 residues; TyrH, tyrosine hydroxylase; BH₄, tetrahydrobiopterin; HXMS, hydrogen/deuterium exchange mass spectrometry; NTA, nitriloacetic acid; MS/MS, tandem mass spectrometry.

mechanisms, using an Fe(IV)O intermediate to hydroxylate the aromatic amino acid substrate (4–6). Each eukaryotic enzyme is a homotetramer with subunits consisting of three domains, an N-terminal regulatory domain, a catalytic domain and a C-terminal tetramerization domain. The catalytic domains are homologous, while the sequence identities among the regulatory domains are low (7).

PheH is strictly regulated to control the metabolism of phenylalanine. Activation by phenylalanine, inhibition by BH₄, and phosphorylation of Ser16 are the three main mechanisms of regulation (3,8–11). Preincubation of PheH with phenylalanine has been reported to increase the activity by more than 100-fold (12). Several studies have suggested that the activation of PheH by phenylalanine is accompanied by conformational changes in the protein. PheH can absorb to phenyl-Sepharose only when phenylalanine is present (13). Treatment of PheH with phenylalanine results in increased fluorescence emission, suggesting a conformational change that exposes a buried tryptophan (14). Pretreatment with phenylalanine and BH₄ have opposite effects on the susceptibility of PheH to limited proteolysis by chymotrypsin (15). There is a lag in the initial rate of tyrosine formation if assays are started by adding enzyme; preincubation of PheH with phenylalanine eliminates the lag while preincubation with BH₄ increases it (12). Based on the opposite effects of phenylalanine and BH₄, Shiman (9,11, 16) proposed a model in which PheH is purified as an inactive form. Treatment with phenylalanine converts the enzyme into the active form while treatment with BH₄ traps the enzyme in a BH₄-PheH complex that is more difficult to activate.

The structural basis for activation of PheH by phenylalanine is not known. The only available structures of PheH with an amino acid substrate bound are ternary complexes consisting of the catalytic domain of human PheH with BH₄ and 3-(2-thienyl)-L-alanine or L-norleucine (17,18). These structures provide no insight into the changes in the regulatory domain associated with phenylalanine or BH₄ binding. Indeed, the isolated catalytic domain does not require activation by phenylalanine (19). The only crystal structure containing the regulatory domain is of a dimeric rat PheH lacking the C-terminal 24 residues of the tetramerization domain; unfortunately, residues 1–18, including the phosphorylation site, are not seen in that structure (20). This structure shows that the N-terminal residues 19–33 extend over the active site pocket. Based on this structure, Jennings et al. (21) proposed that these residues constitute an N-terminal autoregulatory sequence that obstructs the active site in the absence of phenylalanine, and that binding of phenylalanine activates the enzyme by displacing this sequence. While such a model is reasonable based on the known structure, direct evidence is lacking. Moreover, whether activation is due to the localized movement of the N-terminal ~33 residues or to a more global conformational change is unknown.

Hydrogen/deuterium exchange mass spectrometry (HXMS) has proven to be a powerful tool to study the structure and dynamics of proteins in solution (22,23). We describe here the use of HXMS to probe the structural changes accompanying binding of phenylalanine to PheH and the resulting insights into the molecular basis for the regulation of the enzyme.

Experimental Procedures

Materials

Phenyl-Sepharose CL-4B was purchased from GE Healthcare (Piscataway, NJ). Porcine stomach pepsin A was from Worthington Biochemical Co. (Lakewood, NJ). Deuterium oxide (D₂O, 99% D) was from Cambridge Isotope Laboratories (Andover, MA). Pepstatin A and leupeptin were from Peptides Institute, Inc (Osaka, Japan).

Protein purification and preparation

The purification of wild-type rat PheH and the catalytic domain (Δ 117PheH) were described previously (19,24). To remove the ferric iron present in the enzyme when purified, 45 ml concentrated wild-type PheH (about 2 mg/ml) in 50 mM Hepes, pH 7.0, 15% glycerol, 1 μ M pepstatin A and 1 μ M leupeptin was dialyzed overnight against 1 liter of the same buffer plus 50 mM EDTA and 50 mM nitrilotriacetic acid (NTA), pH 7.0, with one buffer change. The dialysis buffer was then changed to 1 liter of the same buffer without EDTA or NTA with three changes over 12 h. The resulting PheH was $\geq$ 90% apoenzyme by atomic absorption spectroscopy (25). PheH activity was determined using a coupled assay with dihydropteridine reductase, measuring the decrease in absorbance at 340 nm upon NADH oxidation (26).

H/D Exchange

All solutions were made anaerobic by applying cycles of vacuum and argon, and the enzyme was kept under argon throughout the exchange reaction to prevent iron oxidation. The day of the experiment, ferrous ammonium sulfate in 1 mM HCl was added to the apoenzyme to achieve a subunit to iron ratio of 1:1 (mole/mole). The H/D exchange reactions were initiated by diluting 15 μ l freshly thawed PheH (about 150 μ g) into 300 μ l of 50 mM Hepes, pH 7.0, in D₂O with or without 5 mM phenylalanine at 25 °C. Twenty μ l samples were taken out with an air-tight syringe at different time points and quenched with 20 μ l ice-cold 100 mM sodium citrate buffer, pH 2.4. The mixture was immediately frozen in liquid nitrogen and stored at -80 °C.

For mass spectrometry, the quenched sample was rapidly thawed, and 2 μ l of a pepsin solution was added to yield a ratio of pepsin to PheH of 1:1 by weight. After 5 min on ice, 20 μ l of the sample was injected onto a Vydac C18 HPLC column (2.1 mm $\times$ 150 mm). The HPLC buffers, column, injection loop and tubing were all immersed in an ice bath. After desalting with 98% 0.1% formic acid, pH 2.4, for 3 min, a 15 min linear gradient of 20–50% 0.1% formic acid in acetonitrile was used to separate the peptides. The flow rate was 200 μ l/min. The outlet of the HPLC was connected to a Thermo Finnigan LCQ DECA XP ion-trap mass spectrometer. All the peptides analyzed eluted after 6 to 12 min with m/z values of 400 to 2000. Each reaction was performed at least twice. Tandem mass spectrometry (MS/MS) under the same conditions using water instead of D₂O was used for peptide identification. The software used to process the MS/MS data was TurboSEQUENT from Thermo Finnigan, version 3.1. Singly, doubly and triply charged peptides were analyzed. The mass spectrometry experiments were all conducted in the Protein Chemistry Laboratory at Texas A&M University.

To determine the extent of back-exchange of hydrogen from H₂O into the deuterated peptides during the analysis, 15 μ l of 200 μ M ferrous PheH was diluted with 300 μ l of 50 mM Hepes, pH 7.0, in H₂O, followed by mixing with 315 μ l of 100 mM sodium citrate buffer (pH 2.4). Pepsin was then added to give a ratio of pepsin to PheH of 1:1 by weight. After pepsin digestion on ice for 5 min, the mixture was immediately frozen with liquid nitrogen and lyophilized. The lyophilized powder was dissolved in 315 μ l of 50 mM Hepes D₂O (pD 7.5) and heated for 30 min at 100 °C. The solution was then incubated at 25 °C for 6 hrs. For mass spectrometry, 20 μ l of the solution was mixed with 20 μ l of 100 mM sodium citrate buffer (pH 2.4). After 5 min on ice, 20 μ l of the sample was injected onto the HPLC, and the amount of deuterium remaining was determined by mass spectrometry.

Data processing

The MS data processed using the Thermo Finnigan Xcalibur software were transferred into HX-Express, a Microsoft Excel-based software for generating a deuterium incorporation curve (27). All of the data in figures are averages of at least two separate runs. After correcting for back-exchange, the kinetics of exchange were fit to equation 1 or equation 2 using software KaleidaGraph (Synergy Software), where N is the fractional exchange over the observed time, and A and B are the fractions of amide hydrogens exchanging with rate constants k_1 and k_2 , respectively.

$$Y=N - Ae^{-k_1t} \quad (1)$$

$$Y=N - Ae^{-k_1t} - Be^{-k_2t} \quad (2)$$

Results

Peptide identification and sequence coverage

Before conducting the H/D exchange experiment, the identities of peptides generated by pepsin digestion of wild-type PheH and of a mutant protein containing only the catalytic and tetramerization domains (Δ 117PheH) were determined by tandem mass spectrometry. For wild-type PheH, a total of 31 peptides could be routinely identified, covering 82% of the protein sequence and evenly spread throughout the sequence (Figure 1). For Δ 117PheH, a total of 28 peptides were used covering 89% of the protein sequence.

H/D exchange of wild-type PheH

To carry out the exchange reactions, a concentrated sample of PheH was diluted into a 20-fold excess of buffered D₂O. To ensure that the analyses used the active ferrous form of the enzyme, the ferric iron found in the enzyme as purified was removed and replaced with ferrous iron under an argon atmosphere beforehand. In addition, the exchange reactions were carried out under argon to prevent oxidation of the iron. Aliquots were removed from the exchange reactions after 30 s to ~4 h and added to ice-cold buffer at pH 2.4 to quench the exchange. The protein was proteolyzed with pepsin, and the resulting peptides were separated by HPLC before

injection into the electrospray mass spectrometer to determine the extent of deuterium incorporation. The time courses of exchange of deuterium from the solvent into individual peptides in PheH are shown in Figure 2 and Figure 3. In all cases the isotopic composition of the peptides during the exchange reaction was consistent with EX2 kinetics (28) for the exchange reaction (results not shown). While the rate constants and extents of exchange varied among the peptides, all had reached a limiting level of exchange after 1 h.

Figure 4 shows the extent of exchange individual peptides after 4 h in the context of the protein structure. The N-terminal regulatory domain and the C-terminal helix show significantly more incorporation of deuterium than the catalytic domain, suggesting that these parts of the protein have more dynamic structures.

The exchange of deuterium into PheH was also determined in the presence of 5 mM phenylalanine to determine the effects of allosteric activation on the structure. The results are shown in Figure 2 and Figure 3 together with the results in the absence of phenylalanine. Several peptides in both the regulatory and catalytic domains exhibit significantly different kinetics of deuterium incorporation in the presence of phenylalanine. In all cases there is an increase in the amount of deuterium incorporation in the presence of phenylalanine. Of the seven peptides from the regulatory domain, two, 39–59 and 82–91, show a large increase in deuterium incorporation in the presence of phenylalanine (Figure 2). Two other peptides, 54–61 and 85–112, show smaller increases; the increased incorporation into these two peptides is most likely due to residues in common with peptides 39–59 and 82–91, respectively, since the effects of phenylalanine on the exchange of those peptides are much larger. The data are consistent with binding of phenylalanine altering the conformation of the regulatory domain to a more exchange-competent form.

The exchange kinetics of a number of peptides in the catalytic domain are also altered in the presence of phenylalanine (Figure 3). Nine peptides in the catalytic domain show a clear increase in total exchange in the presence of phenylalanine: 129–143, 164–174, 205–213, 220–227, 241–248, 249–259, 309–317, 370–376, and 377–386. The exchange kinetics of the three peptides, 129–143, 370–376, and 377–386, are unusual in that the exchange becomes slower in the presence of phenylalanine, but more deuterium is incorporated. All three peptides contain both loop and helical segments. Residues 374–384 form a loop over the active site cavity in the catalytic domain of PheH. The remainder of peptide 370–376 is part of a short helix on the protein surface, while residue 385 is at the beginning of a short β -strand. The behavior of these peptides suggests that the helices at the ends of the loops are extended in the presence of phenylalanine as the helix becomes more flexible.

H/D exchange of $\Delta 117$ PheH

In addition to its role in activating PheH, phenylalanine must bind to the enzyme as a substrate. $\Delta 117$ PheH is a mutant protein lacking the N-terminal regulatory domain. This protein is fully active in the absence of pretreatment with phenylalanine (21,29). Thus, any changes in the deuterium exchange kinetics of $\Delta 117$ PheH in the presence of phenylalanine

can be attributed to catalysis rather than regulation. Consequently the effects of phenylalanine on the kinetics of incorporation of deuterium into peptides of $\Delta 117$ PheH were analyzed. The results are shown in Figure 5. None of the peptides in $\Delta 117$ PheH show the large increases in deuterium incorporation in the presence of phenylalanine exhibited by several of the peptides in the wild-type protein. However, several peptides in $\Delta 117$ PheH do show clear decreases in deuterium incorporation in the presence of phenylalanine: 192–213, 269–284, 300–305, 348–355, and 377–387. In contrast, peptide 446–454 shows an increase in deuterium incorporation in the presence of phenylalanine, although the effect is less than the increases seen in the wild-type protein. The qualitative and quantitative differences in the effects of phenylalanine on the deuterium exchange kinetics of wild-type and $\Delta 117$ PheH provide strong evidence that the changes seen with the former are related to activation by phenylalanine.

Discussion

Analysis of the effects of ligands on the kinetics of incorporation of deuterium from solvent into protein peptide bonds is a powerful probe of the changes in the local dynamics of a protein accompanying binding (30). The exchange into the peptide bonds of folded proteins is typically observed to follow EX2 kinetics (28), in that exchange requires a transient disruption of a backbone hydrogen bond before exchange can occur. This generally involves formation of a more open structure in the vicinity of the peptide. The kinetics of exchange for a peptide are determined by the intrinsic rate constants for exchange of each peptide bond in the open form and the equilibrium constant for formation of the open form. A change in the rate constant for exchange or the extent of exchange is then due to a change in the local protein conformation or dynamics. Increased exchange indicates that the peptide is more frequently in an open or exchange-competent form, while decreased or slower exchange is indicative of a more closed or less dynamic conformation.

The HXMS results presented here establish that binding of phenylalanine to PheH has significant effects on the structure of several regions of the protein. Further interpretation requires consideration of the location of the individual peptides in the structure. X-Ray crystallography has been used to determine the three-dimensional structures of a number of forms of PheH. Unfortunately, no one structure contains the complete protein. The most complete structure is of the ferric form of rat PheH with no ligands bound, but this structure lacks residues 1–18, 137–142, and 428–454 (20). The most catalytically relevant of the available structures is of the ferrous form of the catalytic domain of human PheH with BH_4 and an unnatural amino acid, either norleucine or 2-thienylalanine, bound (18). These two structures show the binding sites of the substrates and the mobile loop containing residues 137–142 that is lacking in the other structures. However, they lack the regulatory domain and the C-terminal 26 residues. The only available structure of PheH with the C-terminus intact is of the ferric form of the human enzyme; no amino acid substrate is bound in this case (31). Still, combining these structures provides a complete structure of PheH and a structural context for interpretation of the present results.

In the absence of ligands, the regulatory domain and the C-terminal helix incorporate more deuterium than the catalytic domain (Figure 4), suggesting that the termini of the protein are more flexible. To date, crystal structures of the aromatic amino acid hydroxylases have only been described for proteins in which either or both the regulatory domain and the C-terminal helix have been removed (17,20,31–34), and expression of the recombinant proteins is often increased upon their removal (19,29,35,36). The N-terminal regulatory domains are also more susceptible to proteolysis than the remainder of the protein (37–40). The present results are consistent with these earlier observations, in that greater flexibility of the regulatory domain would be expected to both hinder crystallization and increase the susceptibility to proteases. Within the catalytic domain, the regions showing the most exchange are in the surface loops containing residues 131–146, 245–250, and 374–385. Peptide 129–143 shows the greatest exchange of any in the absence of phenylalanine. This region is routinely not seen in structures of the aromatic amino acid hydroxylases in the absence of the amino acid substrate (32–34), consistent with it being quite flexible. In TyrH the motion of the corresponding peptide decreases in the presence of a pterin but not in the presence of an amino substrate alone (41).

Because $\Delta 117$ PheH lacks the regulatory domain and does not require pretreatment with phenylalanine for activation, conformational changes in this protein in the presence of the amino acid substrate should be related to catalysis rather than regulation. No structures have been described to date of any of the aromatic amino acid hydroxylases with an amino acid substrate bound in the absence of a pterin. There are a number of differences between the structure of the catalytic domain of PheH with both amino acid and pterin bound and structures with no ligand or only a pterin (17). In the absence of a structure with only an amino acid bound, it is not possible to determine whether these structural changes occur upon binding of the amino acid substrate alone or require both substrates to be present. Figure 6 shows the structure of the catalytic domain of human PheH with BH_4 and norleucine bound (18). Five peptides in $\Delta 117$ PheH show decreased deuterium incorporation in the presence of phenylalanine. As shown in Figure 6, all are located in or near the binding site for the amino acid substrate. Peptide 269–284 is in a surface loop that contains Arg270, which binds the substrate carboxylate, and Thr278, which forms hydrogen bonds with the substrate amino group and one of the carboxylate oxygens (17). Peptide 348–355 is in a helix that contains Ser349; the side chain hydroxyl of this residue forms a hydrogen bond with the other carboxylate oxygen of the substrate. Peptide 192–213 does not contain any residues that directly interact with the substrate. However, residues 203–213 are in a long helix that packs against the helix containing Ser349, suggesting that the decreased movement of residues 348–355 affects the dynamics of residues in peptide 192–213. Peptide 377–387 contains a surface loop that closes over the amino acid when both BH_4 and an amino acid are bound. Val379 on this loop plays a critical role in determining the amino acid substrate specificity of PheH and TyrH (42), confirming that residues on this loop do interact with the substrate, although indirectly. Peptide 300–305 does not contain any residues that interact directly with the amino acid substrate. This peptide incorporates one additional deuterium in the presence of phenylalanine. Because peptide 303–308 does not show any change in the presence of phenylalanine, the additional peptide bond exhibiting exchange in 300–305 is likely to be

between residues 300 and 301 or 301 and 302. These residues are at the end of the helix containing residues 297–311. The preceding helix, 284–295, forms part of the binding site for the amino acid substrate. We were unable to detect residues 285–299 by mass spectrometer. Still, the decreased exchange of peptides 269–284 and 300–305 suggests that the helix between them is affected by the binding of the amino acid substrate. Thus, the decreased deuterium incorporation into these five peptides in the presence of phenylalanine can be attributed to binding of the substrate in the active site. In contrast to the behavior of these five peptides, peptide 446–454 shows increased exchange in the presence of phenylalanine, consistent with increased mobility of this region of the protein. This peptide is at the end of the C-terminal coiled-coil responsible for the tetrameric structure of the PheH. The exchange in this case suggests that some fraying of the end of the helix occurs in the absence of the regulatory domain, and that this increases when the amino acid substrate is bound. Overall, the HXMS results with $\Delta 117$ PheH show that PheH does bind phenylalanine in the absence of BH_4 , and that this results in conformational changes. These changes in deuterium exchange kinetics upon phenylalanine binding are limited to the active site in the absence of the regulatory domain. Since $\Delta 117$ PheH lacks the regulatory domain, they can be ascribed to catalytically relevant events rather than a conformational change related to regulation.

The effect of phenylalanine on the deuterium exchange kinetics of the wild-type protein is very different from that of the catalytic domain alone. A different set of peptides shows altered kinetics, the magnitude of the change is much greater, and an increase in the extent of exchange is seen in all cases. This establishes that the changes in this case reflect conformational changes related to regulation of the protein rather than to catalysis. Figure 7 shows the location in the PheH structure of the peptides showing increased deuterium incorporation in the presence of phenylalanine. The domains are shown in both cartoon and surface renderings for clarity.

Of the peptides in the regulatory domain, peptide 3–11 exchanges nearly 100% within a few minutes, consistent with the very N-terminus of the protein being highly mobile even in the absence of phenylalanine. The first 18 residues are absent from the structure of the combined regulatory and catalytic domains (20); this can be attributed to the dynamic nature of this region of the protein. Peptide 21–38 exchanges ~60% over the same time. Structurally, this peptide is composed of a beta strand from residue 35–38, with the remainder an extended peptide extending over the catalytic domain. The exchange kinetics of this peptide are consistent with its N-terminal 10–11 residues also being very mobile. Thus, the N-terminal ~30 residues are quite dynamic irrespective of the presence of phenylalanine. Peptides 39–59 and 82–91 are the two peptides in the regulatory domain with altered exchange in the presence of phenylalanine. Residues 38–42 interact directly with the catalytic domain; the increased exchange of this peptide is consistent with an altered interaction between the domains in the presence of phenylalanine. These two peptides also contain helices that pack against one another (Figure 7); the increased exchange of peptide 82–91 in the presence of phenylalanine suggests that the interaction between these two helices is partly disrupted when the amino acid binds and that the regulatory domain becomes more open in the presence of phenylalanine.

The peptides in the catalytic domain that show increased exchange in the presence of phenylalanine lie at the interface between the catalytic and regulatory domains (Figure 7). Indeed, the entire interface exhibits increased exchange in the presence of phenylalanine, consistent with an altered interaction between the two domains. Previous studies of PheH have shown that activation by phenylalanine results in an increase in the fluorescence of the enzyme, primarily due to increased solvent exposure of Trp120 (43–45). We were unable to reliably detect a peptic peptide containing residues 113–128 by mass spectrometry. In the surface rendering in Figure 7, these residues are seen as a gray surface patch that interacts directly with the regulatory domain. Including this peptide in those affected by phenylalanine binding makes it clear that the entire interface between the regulatory and catalytic domains becomes more exposed to solvent upon activation of the enzyme. This decreased interaction between the two domains in the activated protein is consistent with the isolated catalytic domain no longer requiring activation.

The extent of the changes in H/D exchange seen in the presence of phenylalanine suggest that more than a simple movement of the N-terminal ~29 residues away from the active site occurs upon activation of the enzyme. The conformational changes that occur upon activation of the related enzyme TyrH upon phosphorylation were recently examined by HXMS (46). In that case the results were consistent with phosphorylation resulting in the N-terminal ~70 residues moving away from the active site upon phosphorylation without any other changes in the structures of the catalytic or regulatory domains. Thus, TyrH can serve as a model for the expected change in deuterium exchange upon movement of an auto-inhibitory N-terminal peptide away from the active site of an aromatic amino acid hydroxylase in the absence of a more global conformational change. TyrH is 46 residues longer than PheH due to a larger regulatory domain, so that peptides 175–190, 210–225, 287–295, 295–299, 355–363, and 419–433 in TyrH correspond to the PheH catalytic domain peptides 129–143, 164–174, 241–248, 249–259, 309–317, 370–376, and 377–386 that are affected by phenylalanine binding. Of these peptides in TyrH, only 295–299 (corresponding to residues 246–253 in PheH) shows increased deuterium incorporation upon phosphorylation. In PheH residues 245–250 make up a loop that lies at the opening to the active site, directly under residues 27–29 of the regulatory domain. This suggests that a simple movement of the N-terminus of PheH away from the active site, similar to that seen upon TyrH upon phosphorylation, would result in increased solvent exposure of residues in peptides 241–248 and 249–259 alone. The observation of altered deuterium exchange in a number of other peptides in both the catalytic and regulatory domain of PheH in the presence of phenylalanine suggests that the conformational changes upon binding of this amino acid are extensive.

The present results cannot establish where phenylalanine binds in order to activate PheH. The question of whether there is an allosteric binding site in addition to the catalytic site is still unsettled. The different effects of phenylalanine binding on the wild-type enzyme and the $\Delta 117$ mutant lacking the regulatory domain suggests that there is another binding site when the regulatory domain is present. Shiman et al. (47) concluded that the phenylalanine that bound to the enzyme to activate it is not available for hydroxylation, so that there must be a binding site other than the active

site. The regulatory domain of PheH has structural similarities to the ACT regulatory domains of allosteric enzymes that bind amino acids (20,48), leading to Kobe et al. (20) to propose that the regulatory binding site is at the interface of the regulatory and catalytic domains, in the vicinity of the beta strand composed of residues 66–69. Such a model is consistent with the present results, in that binding of phenylalanine in the interface of the regulatory and catalytic domains could result in the altered interactions between the two domains detected here.

The present results provide evidence for the proposal that PheH is converted into an open form upon binding phenylalanine. The results support the model that phenylalanine activation displaces the N-terminal autoinhibitory sequence from the active site. This is more than a simple movement of this portion of the protein away from the active site. Rather, a dramatic reorientation of the regulatory and catalytic domains takes place upon phenylalanine activation. PheH is also regulated by phosphorylation of an N-terminal serine residue. Whether this results in the same conformational change as is seen upon phenylalanine activation or a more limited change as is seen with TyrH is presently under investigation.

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Figure 1.

Sequence coverage of PheH. The peptides analyzed in this study are indicated by double-headed arrows. Peptic peptides analyzed for wild-type PheH are in black; peptides from $\Delta 117$ PheH are in magenta. Amino acid residues in the regulatory, catalytic, and tetramerization domains are blue, black, and brown, respectively.

Figure 2. Deuterium exchange kinetics of peptides in the regulatory domain of TyrH in the absence of phenylalanine (○) or in the presence of 5 mM phenylalanine (●) at pH 7 and 25 °C.

Figure 3.

Deuterium exchange kinetics of peptides in the catalytic and tetramerization domains of TyrH in the absence of phenylalanine (○) or in the presence of 5 mM phenylalanine (●) at pH 7 and 25 °C.

Figure 4.

Dynamics of PheH as measured by H/D exchange. The regulatory domain is on the right. The extent of deuterium incorporation after 4 h is indicated by color: blue, $\leq 25\%$; green, 25–50%; orange, 50–75%; red, $>75\%$. The residues in gray were not detected by mass spectrometry. To portray the structure, the structures of the ferrous catalytic domain in the presence of BH_4 and norleucine (pdb code 1mmt) and of the ferric tetrameric catalytic domain (2pah) were aligned with the structure of the combined regulatory and catalytic domains (pdb code 2phm). The regulatory domain from 2phm, the catalytic domain and iron from 1mmt, and the tetramerization helix from 2pah are shown.

Figure 5.

Deuterium exchange kinetics of peptides in $\Delta 117$ TyrH in the absence of phenylalanine (○) or in the presence of 5 mM phenylalanine (●) at pH 7 and 25 °C.

Figure 6. Location in the structure of the catalytic domain of peptides in $\Delta 117$ PheH showing altered (red) deuterium incorporation or no change (cyan) in deuterium incorporation in the presence of phenylalanine. Residues in gray were not detected by mass spectrometry. The structure is that of the catalytic domain of ferrous PheH with tetrahydrobiopterin (BH_4) and norleucine (nle) bound from pdb file 1mmt.

Figure 7.

Location in the structure of the regulatory and catalytic domains of peptides in wild-type PheH with altered deuterium incorporation kinetics in the presence of phenylalanine. The figures are shown with the same orientation, but the catalytic domain is shown in cartoon rendering in the top and surface rendering in bottom, while the regulatory domain is shown in cartoon rendering in both. Residues showing altered deuterium incorporation in the presence of phenylalanine are in red, residues showing no change in deuterium incorporation in the presence of phenylalanine are shown in cyan for the regulatory domain and in light cyan for the catalytic

and tetramerization domains, and residues were not detected by mass spectrometry are shown in gray.