Mutation to Phenylalanine of Tyrosine 371 in Tyrosine Hydroxylase Increases the Affinity for Phenylalanine†

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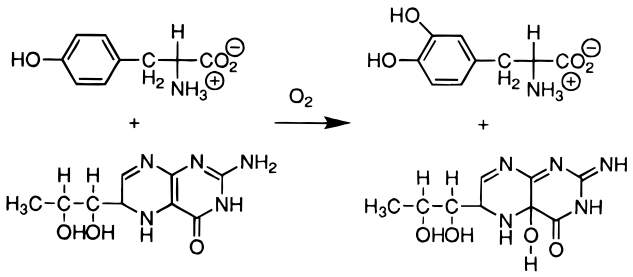
Received July 10, 1998; Revised Manuscript Received September 22, 1998

ABSTRACT: The aromatic amino acid hydroxylases tyrosine and phenylalanine hydroxylase both contain non-heme iron, utilize oxygen and tetrahydrobiopterin, and are tetramers of identical subunits. The catalytic domains of these enzymes are homologous, and recent X-ray crystallographic analyses show the active sites of the two enzymes are very similar. The hydroxyl oxygens of tyrosine 371 in tyrosine hydroxylase and of tyrosine 325 of phenylalanine hydroxylase are 5 and 4.5 Å, respectively, away from the active site iron in the enzymes. To determine whether this residue has a role in the catalytic mechanism as previously suggested [Erlandsen, H., et al. (1997) *Nat. Struct. Biol. 4*, 995−1000], tyrosine 371 of tyrosine hydroxylase was altered to phenylalanine by site-directed mutagenesis. The Y371F protein was fully active in tyrosine hydroxylation, eliminating an essential mechanistic role for this residue. There was no change in the product distribution seen with phenylalanine or 4-methylphenylalanine as a substrate, suggesting that the reactivity of the hydroxylating intermediate was unaffected. However, the K_M value for phenylalanine was decreased 10-fold in the mutant protein. These results are interpreted as an indication of greater conformational flexibility in the active site of the mutant protein.

Tyrosine hydroxylase (TYH)[1] catalyzes the formation of dihydroxyphenylalanine (DOPA) from tyrosine, tetrahydrobiopterin (BH_4), and oxygen (Scheme 1), the first step in the biosynthesis of the catecholamine neurotransmitters (*1*). The enzyme is a tetramer of identical subunits (*2*), each of which contains 498 amino acid residues arranged in three functional domains (*3*−*5*). The regulatory domain extends from the N terminus of the protein to approximately residue 160; the catalytic domain containing the active site residues extends from there to approximately residue 450, and the remaining C-terminal amino acids are responsible for tetramer formation (*4*, *5*). Each subunit contains one non-heme iron which is required for catalytic activity (*6*, *7*). The enzyme phenylalanine hydroxylase (PAH) displays marked functional and structural similarities to TYH. Both enzymes catalyze critical steps in metabolism, as illustrated by the deleterious effects of altered activity. Thus, decreased PAH activity is the major cause of phenylketonuria (*8*), while altered levels of catecholamines have been implicated in hypertension, Parkinsonism, bipolar affective disorder, and schizophrenia (*9*−*12*). Like TYH, PAH also utilizes oxygen

Scheme 1



and tetrahydrobiopterin to hydroxylate an aromatic amino acid, contains non-heme iron, and is a tetramer with three functional domains (*13*, *14*). The sequences of the catalytic domains of PAH and TYH are 75% identical, while there is no homology between the regulatory domains (*15*). On the basis of this high degree of identity, it has been proposed that the catalytic domains of the two hydroxylases are derived from the same ancestral gene (*16*).

In light of the homology of their catalytic domains, one might expect that the two enzymes could carry out the same reactions. Indeed, TYH is a very capable phenylalanine hydroxylase (*17*, *18*). The K_{phe} value of TYH is only 3-fold lower than that of PAH, and the V_{max} value for tyrosine formation is 25% of that of PAH (*19*). PAH and the isolated catalytic domain of PAH are unable to hydroxylate tyrosine to form DOPA, but they do carry out tyrosine-dependent oxidation of tetrahydropterin. Both TYH and PAH are able to hydroxylate a variety of 4-substituted phenylalanines and to hydroxylate the methyl group of 4-methylphenylalanine (*20*, *21*). Thus, the two enzymes share striking functional as well as structural similarities.

† This research was supported in part by NIH Grant GM 47291 and Robert A. Welch Foundation Grant A-1245.

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[1] Abbreviations: DOPA, dihydroxyphenylalanine; TYH, tyrosine hydroxylase; PAH, phenylalanine hydroxylase; 6-MePH_4, 6-methyltetrahydropterin; BH_4, tetrahydrobiopterin; K_{phe}, K_M value for phenylalanine; K_{tyr}, K_M value for tyrosine; K_{MPH4}, K_M value for 6-methyltetrahydropterin; K_{BH4}, K_M value for tetrahydrobiopterin.

10.1021/bi981648f CCC: $15.00
Published on Web 10/29/1998

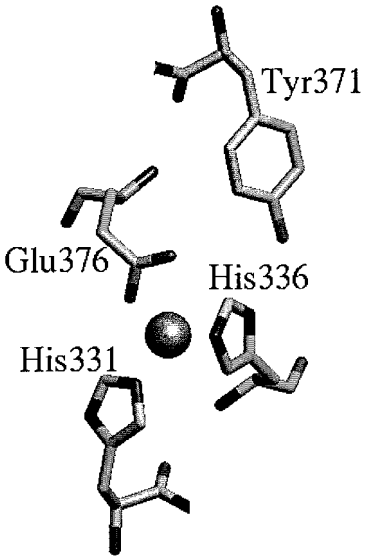


FIGURE 1: Residues near the iron in the active site of rat tyrosine hydroxylase. This figure was prepared using PDB file 1TOH.

The three-dimensional structures of the catalytic domains of TYH (*22*) and PAH (*23*) have recently been determined. The two enzymes are very similar in overall structure and in the configurations of the active sites. In both enzymes, the iron is at the bottom of a cleft, some 10 Å from the protein surface. Both structures have four amino acid residues with side chains within 5 Å of the iron (Figure 1). These include the one glutamyl and two histidinyl residues which were known to coordinate the iron from studies of mutant proteins (*24*, *25*). In addition, there is a conserved tyrosyl residue at position 371 in TYH and position 325 in PAH, the hydroxyl oxygen of which is 4.5 Å from the iron in TYH and 5 Å away in PAH. The proximity of this latter residue to the iron led Erlandsen et al. (*23*) to suggest that it has a role in catalysis. Specifically, by using an analogy to other metalloproteins with tyrosyl residues in the active site, they raised the possibility that it could stabilize a radical during catalysis. To test this hypothesis, we have characterized a mutant protein of TYH containing phenylalanine in place of tyrosine 371.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were custom synthesized on an Applied Biosystems model 380B DNA synthesizer by the Gene Technology Laboratory of the Biology Department of Texas A&M University. 6-Methyltetrahydropterin (6-$MePH_4$) was purchased from B. Schircks (Jona, Switzerland). (6*R*)-Tetrahydrobiopterin was from Calbiochem. Phage M13KO7 was from Pharmacia. Restriction endonucleases were purchased from Promega. Plasmids were purified using kits from Qiagen. Catalase was obtained from Boehringer Mannheim. Heparin−Sepharose was purchased from Pharmacia. *Escherichia coli* strain C41(DE3) was a gift from B. Fox (University of Wisconsin, Madison, WI). *E. coli* strain XL1-Blue from Stratagene was used during DNA subcloning protocols. *E. coli* strain CJ236 from Invitrogen was used for the production of single-stranded uridine-containing DNA. DNA sequencing was carried out by the Gene Technology Laboratory of the Biology Department of Texas A&M University.

Construction of Vectors for Enzyme Expression. Site-directed mutagenesis was carried out according to the protocol of Kunkel et al. (*26*). Plasmid pETYH8, which contains the cDNA for rat TYH inserted into the *Nco*I and *Bam*HI sites of pET23d, with the unique *Nco*I site providing the codon for methionine 1, has been previously described (*19*). Mutagenesis was carried out using an oligonucleotide which encoded a phenylalanine substitution at amino acid 371 (5′-CACGG TG**TTC** TGGTT CACTG TG**GAG** TTCGG GCTAT-3′) and the silent loss of the nearby *Eco*RI site. The coding region of pETYH8Y371F was sequenced to check for unexpected mutations. Plasmid pETYH8Y371F was introduced into competent *E. coli* C41(DE3) cells for production of recombinant proteins.

Bacterial Cell Growth. Bacterial growth was carried out at 37 °C in LB medium containing 100 μg/mL carbenicillin (LB-carb). One isolated colony of C41(DE3) containing pETYH8 or pETYH8Y371F was picked from LB-agar plates (plates contained 100 μg/mL carbenicillin) no more than 1 week after transformation. One colony was used to inoculate 50 mL of LB-carb, and the culture was incubated for 7 h. This culture was used to inoculate a 1 L culture of LB-carb at a ratio of 10 mL per liter. When the A_{600} value of the culture reached 0.8, isopropyl β-D-thioglucanopyranoside was added to a final concentration of 0.25 mM. Incubation was continued for 14 h. Cells were harvested by centrifugation at 5000*g* for 30 min and stored at −70 °C overnight.

Protein Purification. Tyrosine hydroxylase and the TYH Y371F protein were purified as previously described for the wild type enzyme (*27*). The purification protocol consists of cell lysis, ammonium sulfate precipitation (30−45% saturation), and heparin−Sepharose chromatography. Protein concentrations were determined from the absorbance at 280 nm as described previously (*19*).

Assays. The hydroxylation of tyrosine to form DOPA was analyzed with a colorimetric assay which determines the amount of DOPA formed (*28*). Standard conditions for the assay during purification were 400 μM 6-$MePH_4$, 125 μM tyrosine, 10 μM ferrous ammonium sulfate, 2.5 mM dithiothreitol, 60 μg/mL catalase, and 80 mM HEPES-NaOH (pH 7.1) at 30 °C. Steady state kinetic parameters at pH 7.1 or 6.0 with tyrosine as the substrate were determined with varied concentrations of tyrosine and 6-$MePH_4$ or BH_4 under these conditions, using 0.1 M sodium acetate, 0.1 M MES, and 0.2 M Tris-HCl as the buffer. The extent of tyrosine formation from phenylalanine was measured by monitoring absorbance changes at 275 nm due to the production of tyrosine (*29*). The assays contained 400 μM 6-methyltetrahydropterin and varied concentrations of phenylalanine in 80 mM HEPES-NaOH, 5 mM dithiothreitol, and 60 μg/mL catalase (pH 7.1) at 30 °C. All steady state kinetic data were fit directly to the Michaelis−Menten equation using the program Kaleidagraph. The identities of amino acid products with phenylalanine or 4-methylphenylalanine as the substrate were determined by HPLC analysis using the synthetic products as standards, as previously described (*18*, *20*).

RESULTS

We have previously described the expression and purification of recombinant rat tyrosine hydroxylase from *E. coli* (*27*). The Y371F protein was similarly expressed using the

Table 1: Steady State Kinetic Parameters with Tyrosine for Wild Type TYH and TYH Y371F[a]

enzyme	K_{tyr} (μM)[b]	K_{MPH4} (μM)[c]	K_{BH4} (μM)[c]	V_{max} (min^{-1})[b]
pH 7.1				
wild type TYH	51 ± 17	33 ± 7	27 ± 7	151 ± 12
TYH Y371F	65 ± 18	59 ± 10	53 ± 7	215 ± 26
pH 6.0				
wild type TYH	32 ± 7	55 ± 6	ND[d]	165 ± 5
TYH Y371F	71 ± 10	65 ± 12	ND	211 ± 12

[a] The assay measured DOPA formation from tyrosine in 10 μM ferrous ammonium sulfate, 2.5 mM dithiothreitol, 60 μg/mL catalase, 0.1 M sodium acetate, 0.1 M MES, and 0.2 M Tris-HCl at the indicated pH and 30 °C. [b] Determined using 500 μM 6-methyltetrahydropterin and varied concentrations of tyrosine. [c] Determined using 160 μM tyrosine and varied concentrations of the respective tetrahydropterin. [d] ND, not determined.

Table 2: Steady State Kinetic Parameters with Phenylalanine for Wild Type TYH and TYH Y371F[a]

enzyme	K_{phe} (μM)	V_{max} (min^{-1})
wild type TYH	109 ± 20	96 ± 12
TYH Y371F	10 ± 1.4	86 ± 3

[a] The assay measured tyrosine formation from phenylalanine. Conditions were as follows: 400 μM 6-methyltetrahydropterin, 5 mM dithiothreitol, 60 μg/mL catalase, and 80 mM HEPES-NaOH at pH 7.1 and 30 °C.

pET expression system. The mutant protein comprised approximately 15% of the total soluble cell protein and was readily purified using the protocol developed for the wild type enzyme. From 1 L of culture, we obtained 6.6 mg of pure enzyme with a specific activity of 2.9 μmol mg^{-1} min^{-1}, in an overall yield of 50%. This compares well in activity with wild type TYH, for which we obtain 55 mg of enzyme per liter of culture, with a specific activity of 2.1 μmol mg^{-1} min^{-1}.

The high specific activity of the mutant protein clearly establishes that the hydroxyl moiety of phenylalanine 371 is not required for catalysis. As a further probe of its role, steady state kinetic analyses were carried out. The data with tyrosine as the substrate for both TYH Y371F and wild type TYH are shown in Table 1. These steady state kinetic parameters are not significantly different from those for the wild type enzyme at either pH 6 or 7.1. The K_M values for both 6-methyltetrahydropterin and tetrahydrobiopterin increase by at most 2-fold with the mutant protein. The mutant protein does have a slightly higher V_{max} value, consistent with its higher specific activity.

Tyrosine hydroxylase will also catalyze the hydroxylation of phenylalanine (*17*, *18*, *30*), a more chemically difficult reaction than the hydroxylation of the activated phenolic ring of tyrosine. As a probe of the effects of the mutation on the substrate specificity of tyrosine hydroxylase and on the reactivity of the hydroxylating intermediate, steady state kinetic parameters were determined with phenylalanine as the substrate. In contrast to the very modest effects seen with tyrosine as the substrate, the K_M value for phenylalanine of the mutant protein is 10-fold lower than that of the native enzyme (Table 2). The V_{max} value for tyrosine formation from phenylalanine was not changed.

The steady state kinetic parameters in Tables 1 and 2 were determined by measuring the amount of hydroxylated amino acid produced. HPLC was used to determine the relative stoichiometry of the hydroxylated amino acid and oxidized tetrahydropterin with either tyrosine or phenylalanine as the substrate. Both the wild type and the mutant protein produced 1 mol of tyrosine per mole of 6-MePH_4 consumed, in agreement with the stoichiometry shown in Scheme 1. With phenylalanine as the substrate for both the wild type and the mutant enzyme, significantly more pterin was oxidized than amino acid hydroxylated, in agreement with previous analyses (*18*−*20*). However, the coupling of pterin consumption and amino acid hydroxylation was somewhat tighter for the mutant protein, with a relative stoichiometry of 1.3 compared to a value of 1.9 obtained with the wild type enzyme.

When amino acids other than tyrosine are used as substrates for TYH, multiple products often result (*20*). To determine if the mutation had any effect on the regiospecificity of the enzyme, the products generated upon hydroxylation of phenylalanine and 4-methylphenylalanine were determined. With phenylalanine as a substrate for TYH Y371F, the products were 94% tyrosine and 6% 3-hydroxyphenylalanine, compared to 93% tyrosine and 7% 3-hydroxyphenylalanine which were obtained with wild type TYH. With 4-methylphenylalanine as the substrate for TYH Y371F, the products were 4-HOCH_2-phenylalanine (28%), 4-HO-3-CH_3-phenylalanine (17%), and 3-HO-4-CH_3-phenylalanine (55%), while the wild type enzyme gave 30, 15, and 55%, respectively, of each of the same products.

DISCUSSION

The recently determined structures of the catalytic domains of the pterin-dependent enzymes tyrosine hydroxylase and phenylalanine hydroxylase have allowed identification of active site residues (*22*, *23*). In both enzymes, the essential iron atom has three protein-derived ligands, two histidinyl residues and one glutamyl residue. In addition, three water molecules are within 3 Å of the iron, properly positioned to act as ligands. Site-directed mutagenesis experiments had previously identified the two histidinyl ligands as histidines 331 and 336 in rat TYH and histidines 284 and 289 in rat PAH (*24*, *25*). Spectroscopic analyses of both enzymes had previously suggested that a carboxylate was also a ligand to the metal (*31*, *32*). While the presence of these residues in the structures thus confirmed previous results, both structures showed the unexpected presence of a tyrosine residue near the iron. In PAH, the hydroxyl oxygen of tyrosine 325 is 4.5 Å from the iron, while that of the homologous tyrosine 371 is 5 Å from the iron in TYH. The proximity of this residue to the iron prompted Erlandsen et al. (*23*) to propose that it was involved in the catalytic mechanism. Specifically, they proposed that its role is analogous to that of the active site tyrosine in the biphenyl-cleaving extradiol dioxygenase and similar enzymes (*33*). Moreover, they explicitly raised the possibility that this residue stabilizes a radical intermediate during catalysis.

The chemical mechanisms of TYH and PAH are the subjects of active study. For both enzymes, the ferrous form of the bound iron is required (*7*, *34*, *35*). Catalysis does not occur until the amino acid, tetrahydropterin, and oxygen are all bound (*28*, *36*). In the case of TYH, kinetic analyses with alternate substrates and oxygen isotope effects have

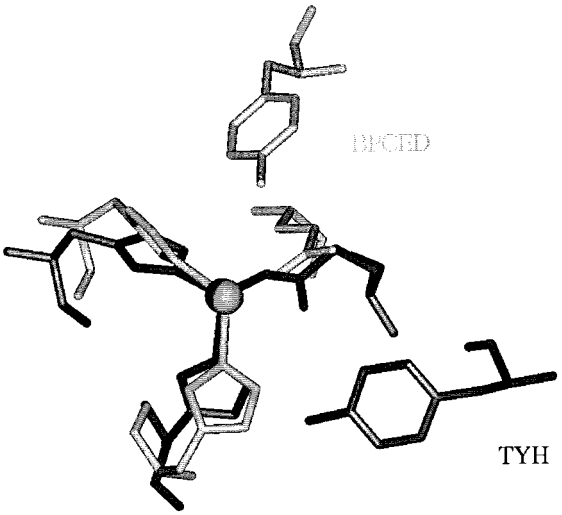
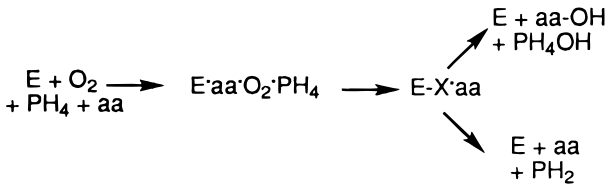


FIGURE 2: Comparison of the active site of rat tyrosine hydroxylase (dark gray) and the biphenyl-cleaving extradiol dioxygenase (BPCED, light gray), showing the metal ligands and the nearby tyrosyl residue. This figure was prepared using PDB files 1TOH and 1HAN.

Scheme 2



shown that the rate-limiting step is formation of the hydroxylating intermediate (*37*, *38*). The identity of this intermediate is not known, although several candidates have been proposed, including a 4a-peroxytetrahydropterin (*39*), a peroxo species involving both the iron and the pterin (*40*), and a high-valence iron oxo species (*38*, *41*). Studies using TYH have led to the conclusion that attack of the hydroxylating intermediate upon the amino acid forms a cationic intermediate (*20*). No mechanistic study to date has invoked a radical involving a protein-derived amino acid residue.

The arrangement of the histidinyl and glutamyl ligands in TYH and PAH is essentially identical to that seen in the biphenyl-cleaving extradiol dioxygenase and isopenicillin N synthase, two mononuclear ferrous iron enzymes (*33*). In Figure 2, the structures of the iron sites in TYH and the extradiol dioxygenase are compared. In addition to the similarities of the iron ligands, both enzymes contain a tyrosyl residue in the vicinity of the iron, although in different orientations with respect to the iron. The hydroxyl of tyrosine 250 of the extradiol dioxygenase is 3.8 Å from the iron, too far to act as a ligand (*42*). Its role in catalysis is unknown. The catalytic mechanism of this enzyme does require an active site base which is as yet unidentified (*33*); histidine 195 has been suggested to have this role (*42*).

The experiments described here directly address whether tyrosine 371 in TYH could act as an active site base or a radical. The results are best discussed in the context of the minimal kinetic mechanism shown in Scheme 2, which is consistent with previous studies of this enzyme (*20*, *28*, *37*, *38*). All three substrates must bind to form the quaternary complex before any chemistry occurs. The hydroxylating intermediate then is formed in the rate-limiting step. This species can react with the amino acid substrate or break down unproductively. Thus, a specific amino acid residue could be involved in binding substrate, in formation of the hydroxylating intermediate, or in determining its fate once it forms.

With tyrosine as the substrate, the TYH Y371F protein is fully active. All the kinetic parameters are within a factor of 2 of those for the wild type enzyme. The lack of a significant effect on the K_M values for the substrates, or more rigorously on the *V*/*K* values, rules out the possibility of the hydroxyl moiety of tyrosine 371 having a critical role in substrate binding. The lack of a significant effect on the V_{max} value establishes that the rate of formation of the hydroxylating intermediate is not significantly perturbed. The fact that tetrahydropterin oxidation and tyrosine hydroxylation remain tightly coupled with the mutant protein rules out a large change in the nature of the hydroxylating intermediate.

The product distribution with phenylalanine or 4-methylphenylalanine as the substrate was used as a more sensitive probe of the reactivity of the hydroxylating intermediate. The partitioning between amino acid hydroxylation and unproductive decomposition of the hydroxylating intermediate is sensitive to the identity of the amino acid substrate, in that electron-withdrawing substituents on the aromatic ring decrease the amount of hydroxylation (*20*). Tyrosine is a relatively activated substrate toward electrophilic reactions, so a decrease in the reactivity of the hydroxylating intermediate might not be noticed with this substrate. In contrast, with phenylalanine as the substrate, the rates of hydroxylation and unproductive breakdown of the intermediate are more closely balanced. The Y371F protein is as effective at hydroxylating phenylalanine as the wild type enzyme, if not more so, ruling out any decrease in the reactivity of the hydroxylating intermediate in this protein. An alternative probe of the reactivity of the hydroxylating intermediate is the product distribution seen when multiple hydroxylated products are formed. With phenylalanine as the substrate for the wild type enzyme, the products are both tyrosine and 3-hydroxyphenylalanine, with the former being the predominant product (*18*). The product distribution with the mutant protein was indistinguishable from that for the wild type enzyme. This is consistent both with a lack of change in the reactivity of the hydroxylating intermediate and with no change in the relative orientation of the amino acid with respect to the hydroxylating species. The product distribution with 4-methylphenylalanine was used as a further test of reactivity. With this substrate, both aromatic and benzylic hydroxylation occur (*20*). Again, the product distribution seen with the mutant protein was not significantly different from that seen with the wild type enzyme.

These results rule out both stabilization of a radical and action as an active site base as essential roles for the hydroxyl moiety of tyrosine 371. However, our results do not show that the mutant protein is indistinguishable from the wild type enzyme. The Y371F protein differs from the wild type enzyme in having a 10-fold lower K_{phe} value and a 10-fold higher *V*/*K* value for phenylalanine hydroxylation. Thus, the mutant protein is a more effective phenylalanine hydroxylase than the wild type enzyme. We have previously analyzed the effects of the regulatory domains of TYH and PAH on the kinetic parameters and substrate specificities of the

Table 3: Steady State Kinetic Parameters for Phenylalanine Hydroxylation by TYH, PAH, and TYH Mutant Enzymes

enzyme	K_{phe} (μM)	V/K_{phe} (μM^{-1} min^{-1})	residue at position 371 (TYH numbering)
TYH[a]	109	1.05	tyrosine
PAH[b]	300	1.24	tyrosine
PAH catalytic domain[b]	145	3.46	tyrosine
TYH catalytic domain[b]	15	5.33	tyrosine
TYH Y371F[a]	10	8.6	phenylalanine

[a] This study. [b] Reference *19*.

catalytic domains (*19*). These experiments showed that loss of the regulatory domains decreased the relative specificity of both enzymes for their native substrates. Table 3 contains kinetic data for TYH, PAH, and the catalytic domains of each enzyme from that study. Using the V/K_{phe} value as the sole criterion, TYH Y371F is the best phenylalanine hydroxylase in the series. The earlier results were interpreted as evidence of increased conformational flexibility of the active site in the absence of the regulatory domain. The increased flexibility resulted in a relaxed amino acid substrate specificity. A similar increase in active site flexibility due to the Y371F mutation would provide a rationale for the increased ability to hydroxylate phenylalanine. The active sites of both TYH and PAH are quite hydrophobic. The phenyl side chain of a phenylalanyl residue would be more readily accommodated in such a hydrophobic environment than the phenolic side chain of a tyrosyl residue. This could lead to more conformational options for the mutant protein, allowing it to more readily bind the nonphysiological substrate.

In summary, the results presented here directly address previously proposed roles for tyrosine 371 in TYH. They rule out a direct role of this residue in catalysis or binding, instead suggesting that this residue contributes to the overall active site structure.

ACKNOWLEDGMENT

We acknowledge the technical expertise of Ellen Provin and Natalie Unruh of the Institute of Developmental and Molecular Biology of Texas A&M University for the synthesis of custom oligonucleotides and DNA sequencing.

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BI981648F