Site-Directed Mutants of Charged Residues in the Active Site of Tyrosine Hydroxylase†

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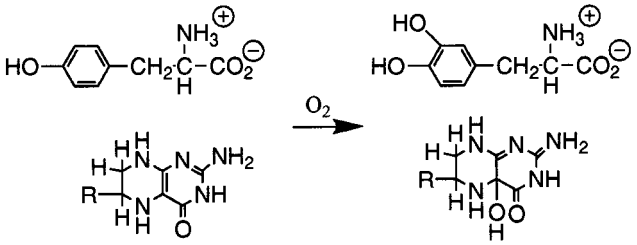
ABSTRACT: The active site of tyrosine hydroxylase consists of a hydrophobic cleft with an iron atom near the bottom. Within the cleft are several charged residues which are conserved across the family of pterin-dependent hydroxylases. We have studied four of these residues, glutamates 326 and 332, aspartate 328, and arginine 316 in tyrosine hydroxylase, by site-directed substitution with alternate amino acid residues. Replacement of arginine 316 with lysine results in a protein with a K_{tyr} value that is at least 400-fold greater and a V/K_{tyr} value that is 4000-fold lower than those found in the wild-type enzyme; substitution with alanine, serine, or glutamine yields insoluble enzyme. Arginine 316 is therefore critical for the binding of tyrosine. Replacement of glutamate 326 with alanine has no effect on the K_M value for tyrosine and results in a 2-fold increase in the K_M value for tetrahydropterin. The V_{max} for DOPA production is reduced 9-fold, and the V_{max} for dihydropterin formation is reduced 4-fold. These data suggest that glutamate 326 is not directly involved in catalysis. Replacement of aspartate 328 with serine results in a 26-fold higher K_M value for tyrosine, a 8-fold lower V_{max} for dihydropterin formation, and a 13-fold lower V_{max} for DOPA formation. These data suggest that aspartate 328 has a role in tyrosine binding. Replacement of glutamate 332 with alanine results in a 10-fold higher K_M value for 6-methyltetrahydropterin with no change in the K_M value for tyrosine, a 125-fold lower V_{max} for DOPA formation, and an only 3.3-fold lower V_{max} for tetrahydropterin oxidation. These data suggest that glutamate 332 is required for productive tetrahydropterin binding.

Tyrosine hydroxylase (TyrH)[1] is the rate-limiting enzyme in the biosynthesis of catecholamine neurotransmitters; it catalyzes the hydroxylation of tyrosine to form L-DOPA (Scheme 1), a precursor of the neurotransmitters dopamine, norepinephrine, and epinephrine. Two closely related enzymes, phenylalanine hydroxylase (PheH) and tryptophan hydroxylase (TrpH), are also rate-limiting enzymes in critical metabolic pathways (phenylalanine catabolism and serotonin biosynthesis). The three enzymes share many traits both structural and functional. All three catalyze the hydroxylation of an aromatic ring, utilizing tetrahydrobiopterin, oxygen, and a non-heme iron atom (*1*−*3*). All are tetrameric in structure. The 330 C-terminal amino acids of all three are 60−80% identical in sequence. A variety of approaches have established that each subunit of TyrH and PheH consists of

Scheme 1



a catalytic domain, an N-terminal regulatory domain, and a C-terminal tetramerization domain (*4*, *5*). While characterization of TrpH has lagged well behind that of these two enzymes, the isolated catalytic domain of TrpH has been purified in an active form and shown to be a monomer (*6*). It has been proposed that the catalytic regions of the three enzymes evolved from the same ancestral gene (*7*, *8*).

Recently, the three-dimensional structures of the catalytic domains of both TyrH (*9*) and PheH (*10*) have been elucidated by X-ray crystallography. In both enzymes, the active site is a hydrophobic cleft consisting of four helices. The active site iron atom is located near the bottom of this cleft. Along the sides of the cleft are several charged amino acid residues which are conserved among the three hydroxylases (Figure 1). Three of these residues [histidine 331, histidine 336, and glutamate 376 (*11*, *12*)] are already known to be ligands to the iron atom. In this study, we focused on the remaining four charged conserved residues, the glutamates

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[1] Abbreviations: DOPA, dihydroxyphenylalanine; IPTG, isopropyl β-D-thioglucanopyranoside; TyrH, tyrosine hydroxylase; PheH, phenylalanine hydroxylase; TrpH, tryptophan hydroxylase; 6-MPH$_4$, 6-methyltetrahydropterin; K_{tyr}, K_M value for tyrosine; K_{6-MPH_4}, K_M value for 6-methyltetrahydropterin; LB-carb, Luria-Bertani broth with 100 μg/mL carbenicillin; LB-amp, Luria-Bertani broth with 100 μg/mL ampicillin.

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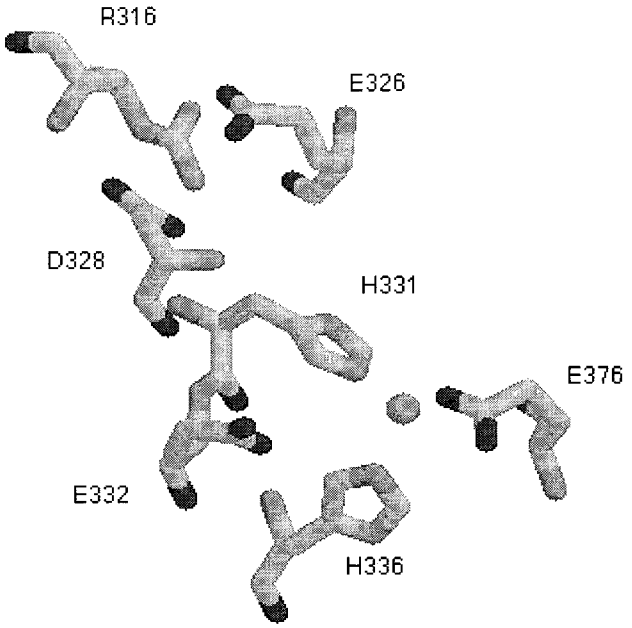


FIGURE 1: Conserved charged residues in the active sites of tyrosine hydroxylase and phenylalanine hydroxylase. The figure was constructed using PDB file 1TOH.

at position 326 and 332, the aspartate at position 328, and the arginine at position 316 of tyrosine hydroxylase.

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 was purchased from Schircks Laboratories. Phage M13KO7 was from Pharmacia Biotech Inc. Restriction endonuclease *Nar*I was purchased from Promega Corp.; *Sph*I, *Bst*UI, and *Ban*II were from New England Biolabs Inc. Plasmids were purified using kits from Qiagen Inc. Leupeptin, pepstatin, and catalase were obtained from Boehringer Mannheim Corp. Sheep dihydropteridine reductase and NADH were from Sigma Chemical Corp. Heparin-Sepharose and DEAE-Sepharose Fast Flow were purchased from Pharmacia Biotech Inc. *Escherichia coli* strain C41(DE3), a strain derived from BL21(DE3) which grows to a higher cell density (*13*), was a gift from B. Fox (University of Wisconsin, Madison, WI). *E. coli* strain XL1-Blue was obtained from Stratagene USA and was used during DNA subcloning protocols. *Pfu* DNA polymerase was obtained from Stratagene USA. *E. coli* strain CJ236 was obtained from Invitrogen Corp. and 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.

Design of Vectors for Enzyme Expression. Site-directed mutagenesis was carried out according to the protocol of Kunkel et al. (*14*) for E326A and for the R316 mutations. Mutagenesis was performed using the Stratagene QuikChange kit for E332A and for the D328 mutations. Plasmid pETYH8, which contains the cDNA for rat TyrH inserted into the *Nco*I and *Bam*HI sites of pET23d with the unique *Nco*I site at the position encoding methionine 1, has been previously described (*15*) and was used as the template for mutagenesis

Table 1: Oligonucleotides Used To Create Mutations in Tyrosine Hydroxylase[a]

mutation	oligonucleotide	selection
R316A	5′-tgcac ccagt atatc **gcgca** tgcct cctca cct-3′	gain of a *Sph*I site
R316S	5′-caccc agtat atc**tc** gcatg cctcc tcac	gain of a *Sph*I site
R316Q	5′-caccc agtat atc**ca** gcatg cctcc tcac-3′	gain of a *Sph*I site
R316K	5′-caccc agtat atc**aa** g catg cctcc tcac-3′	gain of a *Sph*I site
E326A	5′-cctat gcatt cacc**g gcgcc** ggact gctgc cat-3′	gain of a *Nar*I site
D328A	5′-cacct gagcc **ggcat** gctgc catg-3′	gain of a *Sph*I site
D328S	5′-tcacc t**gagc cctct** tgctg ccatg ag-3′	gain of a *Ban*II site
D328N	5′-cacct **gagcc caact** gctgc catg-3′	gain of a *Ban*II site
E332A	5′-ctgct gcca**c gcgc**t gttgg gac-3′	gain of a *Bst*UI site

[a] Oligonucleotides used for E326 and R316 were actually the reverse and complement of those noted here; mutagenesis using pET23 requires that the mutagenic oligonucleotide bind to the noncoding strand. Oligonucleotides used for E332A, D328N, and D328S were the above oligonucleotides and a complementary oligonucleotide; the Stratagene QuikChange kit utilizes oligonucleotides binding to both strands of the template. These sequences are given for ease of comparison to the wild-type sequence. Mutagenic codons are indicated in bold type, and new restriction sites are underlined.

by either method. Mutagenesis was carried out using the oligonucleotides described in Table 1. Mutated plasmids were detected by electrophoretic analysis of restriction digests of plasmids using the enzymes indicated in Table 1. The coding regions of all plasmids which met this criterion were then sequenced to confirm the desired mutation and to identify unexpected mutations.

Bacterial Cell Growth. Plasmids were introduced into competent *E. coli* C41(DE3) and/or BL21(DE3) cells. Small cultures of each were grown for storage as 15% glycerol permanent stocks at −80 °C. Thereafter, cells were streaked onto LB-agar plates (containing 100 μg/mL carbenicillin) for use no earlier than 1 week before growth in liquid culture. For growth of bacteria expressing mutant enzymes, various expression conditions were screened. In each case, the conditions yielding the most enzyme are reported here.

For cells containing pETYHE326A, growth was carried out at 30 °C. Bacteria were grown in LB medium containing 100 μg/mL carbenicillin (LB-carb). One colony of C41(DE3) containing pETYHE326A was picked from LB-agar plates and used to inoculate 20 mL of LB-carb; the culture was incubated for 9 h. This 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 (IPTG) was added to a final concentration of 0.5 mM. Incubation was continued for 12 h. Cells were harvested by centrifugation at 5000*g* for 30 min and stored at −70 °C overnight.

For cells containing pETYHR316A, -R316S, -R316Q, and -R316K, growth was initially carried out under various conditions. Bacteria were grown in LB-carb. All inocula were grown at 37 °C; a colony was picked and used to start a 20 mL culture, which was grown for 12 h. Inocula were added to 1 L of LB-carb at a ratio of 10 mL per liter. The large cultures were immediately moved to incubators at the desired final temperatures of 15−37 °C. When the culture reached an A_{600} value of 0.7, IPTG was added to a final concentration of 0.5 mM. Aliquots were removed periodically within 3−32 h depending on the temperature, and the amount of mutant

protein present was determined by PAGE. For purification, R316KTyrH, the only arginine 316 mutant protein that could be obtained for further study, was grown in the following manner. One colony of BL21(DE3) cells containing pETYHR316K was picked from a plate to inoculate 20 mL of LB-carb; the culture was incubated at 37 °C for 7 h. This culture was used to inoculate 1 L of LB medium containing 100 μg/mL ampicillin (LB-amp) at 37 °C at a ratio of 12 mL per liter, and the fresh culture was then moved to a 22 °C incubator. When the A_{600} value of the culture reached 0.5, IPTG was added to a final concentration of 0.25 mM. Incubation was continued for 12 h. Cells were harvested by centrifugation at 5000*g* for 30 min and stored at −70 °C overnight.

For cells containing pETYHE332A, preinduction growth was carried out at 37 °C and postinduction growth at 18 °C in LB-amp. One colony of BL21(DE3) cells containing pETYHE332A was picked from a plate and used to inoculate 20 mL of LB-amp; the culture was incubated at 37 °C for 5.5 h. This culture was used to inoculate a 1 L culture of LB-amp at a ratio of 15 mL per liter. This culture was maintained at 37 °C until the A_{600} value reached 0.3, after which the flask was moved to an 18 °C incubator. When the A_{600} value of the culture reached 0.7, IPTG was added to a final concentration of 0.25 mM. Incubation was continued for 12 h. Cells were harvested by centrifugation at 5000*g* for 30 min and stored at −70 °C overnight.

For cells containing pETYHD328A, -D328N, and -D328S, initial growths were carried out at several different temperatures in LB-amp. One colony of BL21(DE3) cells containing plasmid was picked from a plate and used to inoculate 20 mL of LB-amp; the culture was incubated at 37 °C for 6 h. This inoculum was used to seed a 1 L culture of LB-amp at a ratio of 15 mL per liter. This culture was incubated at 37 °C until the A_{600} value reached 0.2, after which the flask was moved to a separate incubator at 12, 16, 18, or 20 °C. When the A_{600} value of the culture reached 0.4 for the 12 °C growth or 0.7 at 16, 18, or 20 °C, IPTG was added to a final concentration of 0.25 mM. For purification of pETYHD328S, the cells were grown at 16 °C for 18 h after induction. Cells were harvested by centrifugation at 5000*g* for 30 min and stored at −70 °C overnight.

Protein Purification. Wild-type rat tyrosine hydroxylase, R316KTyrH, and E326ATyrH were purified as previously described for the wild-type enzyme (*16*). The purification protocol consists of cell lysis by sonication, polyethyleneimine precipitation, ammonium sulfate precipitation (30−50% saturation), and heparin-Sepharose chromatography. EDTA is included at a concentration of 75 μM as a protease inhibitor in most buffers, but is removed from pure enzyme, because it increases the rate of autoxidation of tetrahydropterin. At 75 μM, it does not affect the iron content of wild-type TyrH. Therefore, after purification wild-type TyrH and E326ATyrH were dialyzed versus 50 mM Tris-HCl, 10% glycerol, 1 μM leupeptin, and 1 μM pepstatin (pH 7.0) to remove EDTA. R316KTyrH was eluted from heparin-Sepharose with buffer lacking EDTA. E332ATyrH was purified using the published protocol, the exceptions being that 2.5% streptomycin sulfate was used instead of 0.008% polyethyleneimine to remove nucleic acids and that the enzyme was eluted from the heparin-Sepharose column in buffer lacking EDTA. D328STyrH was purified using the published protocol, with the addition of a second chromatography step. The proteins in the heparin-Sepharose eluate were precipitated by adding ammonium sulfate to 60% saturation, and then redissolved to give 1 mg/mL protein in 50 mM Tris-HCl, 10% glycerol, 75 μM EDTA, 1 μM leupeptin, and 1 μM pepstatin (pH 7.0). The sample was dialyzed versus the same buffer containing 75 mM ammonium sulfate and applied to a column of DEAE-Sepharose Fast Flow (1.0 cm × 7 cm). The column was washed until the A_{280} value of the effluent was 0.05, and D328STyrH was eluted with a 0 to 1.0 M NaCl gradient in 50 mM Tris-HCl, 10% glycerol, 1 μM leupeptin, and 1 μM pepstatin (pH 7.0). Electrophoresis on denaturing 8% polyacrylamide gels (*17*) was used to estimate expression levels at early stages of the isolation and purity at later stages. At early stages of the purification, the protein concentration was determined with the Bio-Rad protein assay; after chromatography, protein concentrations were assessed using the absorbance at 280 nm (*16*).

Assays. Wild-type TyrH is inhibited by tyrosine at concentrations above 100 μM, but only in the presence of the physiological substrate tetrahydrobiopterin. To avoid this complication, 6-methyltetrahydropterin was used in all enzyme assays instead.

The hydroxylation of tyrosine to form DOPA was assessed with a colorimetric end-point assay which determines the amount of DOPA formed (*18*). Standard conditions for the assay were 50 mM HEPES/NaOH, 100 μg/mL catalase, 10 μM ferrous ammonium sulfate, 125 μM tyrosine, and 300 μM 6-MPH_4 at pH 6.7 and 32 °C. Wild-type TyrH assays were carried out for 2 min; the mutant proteins were assayed for 5 min. For determination of steady-state kinetic parameters, the concentration of 6-MPH_4 was varied from 5 μM to 1.3 mM, or the concentration of tyrosine was varied from 5 μM to 2.75 mM.

With some alternate substrates, the aromatic amino acid hydroxylases oxidize more moles of tetrahydropterin substrate than they hydroxylate amino acid substrate. Because of the possibility that a mutant TyrH likewise might produce more of one product than the other, rates of dihydropterin production from tetrahydropterin were determined. Using a coupled assay with dihydropterin reductase, the decrease in absorbance at 340 nm due to NADH oxidation was monitored (*19*, *20*). The standard assay contained 125 μM tyrosine, 300 μM 6-MPH_4, 80 mM HEPES/NaOH, 50 μg/mL catalase, 200 μM NADH, 10 μM ferrous ammonium sulfate, and 0.2 unit/mL sheep dihydropteridine reductase at pH 6.7 and 32 °C. Because of the difficulty of accurately measuring enzymatic dihydropterin production at high levels of tetrahydropterin due to background autoxidation, the V_{max} value for E332ATyrH was determined by performing assays at a 6-MPH_4 concentration equal to its K_M value (255 μM) and 200 μM tyrosine and multiplying the velocity obtained by 2. The V_{max} values for dihydropterin formation by E326ATyrH and D328STyrH were determined by performing assays with 300 μM 6-MPH_4 and varying the tyrosine concentration; for E326ATyrH, the range was 10−350 μM, and for D328STyrH, the range was 100 μM to 2.75 mM. Steady-state kinetic data were fit directly to eq 1 using the program Kaleidagraph.

$$v = VS/(K_M + S) \quad (1)$$

Table 2: Steady-State Kinetic Parameters for Active Site Mutants of Tyrosine Hydroxylase

enzyme	K_{tyr} (μM)	K_{6-MPH_4} (μM)	V/K_{tyr}[a] (μM^{-1} min^{-1})	V_{max} for DOPA formation (min^{-1})[b]	V_{max} for dihydropterin formation (min^{-1})[c]	DOPA/dihydropterin[d] × 100
TyrH	25 ± 5	26 ± 8	6.6 ± 1.4	150 ± 14	165 ± 11	91 ± 10
R316K	>10000	52 ± 28[e]	0.0018 ± 0.00012	nd[f]	nd	35 ± 2.5[g]
E326A	21 ± 2	52 ± 7	2.1 ± 0.3	16.8 ± 1.1	43.7 ± 5.2	38 ± 5.2
D328S	641 ± 175	10.3 ± 2.9	0.032 ± 0.009	11.9 ± 0.6	20.6 ± 2.1	58 ± 6.6
E332A	22 ± 3	255 ± 67	2.2 ± 0.3	1.2 ± 0.2	48.9 ± 0.5	2.4 ± 0.4

[a] Based on the rate of dihydropterin formation. [b] The assay measured the extent of DOPA formation from tyrosine. Assays were carried out in 50 mM HEPES/NaOH, 100 μg/mL catalase, and 10 μM ferrous ammonium sulfate at pH 6.7 and 32 °C, as described in Experimental Procedures. [c] The assay measured 6-methyldihydropterin formation from 6-methyltetrahydropterin. Assays were carried out in 80 mM HEPES/NaOH, 100 μg/mL catalase, 200 μM NADH, 10 μM ferrous ammonium sulfate, and 0.1 unit/mL sheep dihydropteridine reductase at pH 6.7 and 32 °C, as described in Experimental Procedures. [d] V_{max} for DOPA formation/V_{max} for dihydropterin formation × 100. [e] Determined at 0.6 mM tyrosine. [f] Not determined. [g] Determined at 1.66 mM tyrosine.

RESULTS

Overexpression and Purification of Mutant Proteins. Most previous descriptions of the production of recombinant TyrH have utilized *E. coli* strain BL21(DE3). The strain C41(DE3) has been reported to grow for longer times without loss of plasmid to degradation than does BL21(DE3) (*13*). We have found that C41(DE3) cells expressing wild-type TyrH can be grown to cell densities with A_{600} values of 6.0−7.0, yielding 10 times more cells upon harvest than a comparable culture with BL21(DE3) cells. For wild-type TyrH, we obtain approximately 60 mg of enzyme/L of culture.

Each of the conserved residues (arginine 316, glutamate 326, glutamate 332, and aspartate 328) was replaced with alanine by site-directed mutagenesis. Initially, the cells were grown under the conditions used for the wild-type enzyme. The mutant protein E326ATyrH was expressed at a level comparable to that of the wild-type protein in C41(DE3), comprising approximately 25% of the total soluble cell protein; 50% of the protein precipitated in the ammonium sulfate step, and was therefore easily purified. From 1 L of culture, we obtained 30 mg of pure enzyme. However, none of the other mutant proteins were expressed in soluble form in C41(DE3) cells. Consequently, strain BL21(DE3) was used instead.

The mutant protein E332ATyrH was readily expressed in abundance in BL21(DE3) cells. It proved to be possible to obtain soluble enzyme when cells were grown at 18 °C. From 1 L of culture, we purified 24 mg of enzyme.

D328ATyrH was also expressed well, but it was insoluble at every temperature and incubation time tested. Consequently, aspartate 328 was replaced with several different residues in an effort to obtain active and soluble mutant protein. With both D328STyrH and D328NTyrH, a small amount of soluble enzyme was produced when cells were grown at 12 or 15 °C. Only D328STyrH was chosen for further study and purified to homogeneity. Because of the relatively low level of expression of the protein, a DEAE-Sepharose chromatography step was added to the purification protocol. From 12 L of culture, approximately 7 mg of enzyme was isolated.

Replacement of arginine 316 with alanine similarly did not yield soluble protein under any growth conditions, although the level of expression at 37 °C was satisfactory. The R316S- and R316QTyrH proteins behaved similarly, being expressed at high levels if growth was carried out at 37 °C. If growths were carried out at lower temperatures, no proteins with the correct molecular mass could be detected with any of these three mutant proteins. Only when arginine 316 was replaced with lysine was it possible to obtain soluble mutant protein. In this case, the cells had to be grown at 22 °C. From 4 L of culture, we purified 8 mg of R316KTyrH.

Steady-State Kinetic Analyses of Mutant Enzymes. Steady-state kinetic analyses were carried out for each of the purified mutant proteins by measuring separately the rates of formation of both products, DOPA and dihydropterin. The respective steady-state kinetic parameters are listed in Table 2 along with the parameters for the wild-type enzyme.

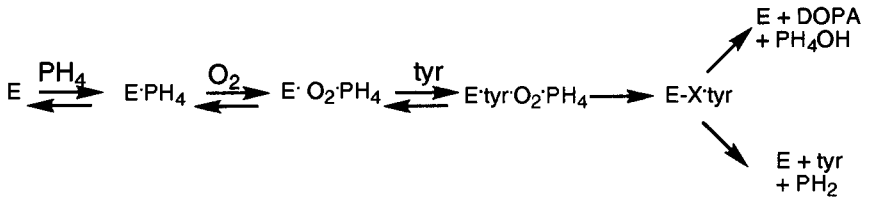
The mutant protein R316KTyrH was almost totally devoid of activity. It was necessary to use micromolar concentrations of enzyme to accurately determine the very low rates of DOPA and dihydropterin formation. The rate of DOPA formation depended directly on the tyrosine concentration over the entire concentration range used. The low solubility of tyrosine limited the tyrosine concentration in the assays to 2.75 mM. No evidence for saturation behavior was seen, indicating that the K_{tyr} value for this protein is at least 10 mM. The V/K_{tyr} value could be determined from the dependence of the velocity on the tyrosine concentration. The value is decreased from the wild-type value by 4 orders of magnitude. The K_{6-MPH_4} value for R316KTyrH was determined at a tyrosine concentration of 0.6 mM; the resulting value is only slightly higher than that for the wild-type enzyme. The rate of DOPA formation is decreased about 3-fold more than the rate of dihydropterin production.

Replacement of glutamate 326 with alanine has only moderate effects on the K_M values for tyrosine and 6-MPH_4. The K_{tyr} value for E326ATyrH is the same as that for wild-type TyrH, while the K_{6-MPH_4} value is about twice the wild-type value. The effect of the mutation on the V_{max} value depends on whether the rate of formation of DOPA or of dihydropterin is measured. E326ATyrH is 9-fold less active than the wild-type enzyme in the production of DOPA from tyrosine, and 4-fold less active in the production of dihydropterin.

Replacement of aspartate 328 with serine by mutagenesis has a more dramatic effect on kinetic parameters. The K_{tyr} value is almost 26 times higher than that for wild-type TyrH, while the K_{6-MPH_4} value is slightly lower than that for the wild-type enzyme. The mutant enzyme D328STyrH has lowered V_{max} values in both activities; for DOPA formation, the V_{max} value is 13-fold lower, and for dihydropterin formation, it is decreased 8-fold.

Mutagenesis of glutamate 332 to an uncharged alanine residue also resulted in a mutant protein with lower activity.

Scheme 2



While the K_{tyr} value is comparable to the one for wild-type TyrH, the K_{6-MPH_4} value is almost 10 times higher than the wild-type value. The V_{max} for tyrosine hydroxylation is reduced 125-fold compared to that of TyrH, while that for dihydropterin production is only decreased 5-fold.

DISCUSSION

The homology of the catalytic domains of phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase and the similarities of the reactions these enzymes catalyze suggest that their catalytic mechanisms are similar if not identical. Despite a great deal of study, the understanding of these chemical mechanisms is still quite limited. At present, tyrosine hydroxylase may be the best understood mechanistically of the pterin-dependent hydroxylases. Study of phenylalanine hydroxylase has been complicated by the allosteric properties of that enzyme (*21*−*23*), while it has only very recently been possible to obtain sufficient amounts of tryptophan hydroxylase for mechanistic studies (*6*). The present understanding of the kinetic mechanism of tyrosine hydroxylase is summarized by the mechanism depicted in Scheme 2. Tetrahydropterin is the first substrate to bind, followed by oxygen; tyrosine is the final substrate to bind (*18*). Because oxygen binds in a rapid equilibrium fashion, there is no K_M value for oxygen. The enzyme does not catalyze a reaction between tetrahydropterins and oxygen in the absence of an amino acid substrate, suggesting that a conformational change occurs after the amino acid binds which is required for initiation of catalysis. The rate-limiting step in catalysis is the formation of the hydroxylating intermediate (X in Scheme 2); this conclusion is supported by studies with alternate amino acid substrates and by kinetic isotope effects (*18*, *20*, *24*). The hydroxylating intermediate then delivers an oxygen atom to the amino acid; the pterin product of this reaction is 4a-hydroxypterin, as shown in Scheme 1. Alternatively, the hydroxylating intermediate can break down unproductively, resulting in production of dihydropterin without concomitant hydroxylation of the amino acid. This unproductive reaction is only significant with the wild-type enzyme when nonphysiological substrates are used (*6*, *25*, *26*). The degree to which unproductive breakdown of the hydroxylating intermediate occurs reflects a balance between its rate of decay and the rate of oxygen atom transfer to the amino acid (*26*).

Until recently, very little could be said about the involvement of any particular amino acid residue in the chemical mechanism of any of the pterin-dependent hydroxylases. Although it is reasonable that residues which are conserved in TyrH, PheH, and TrpH are candidates for catalytically important residues, the high proportion of conserved amino acid residues has limited the insights obtained from sequence comparison. For example, Quinsey et al. mutated several such residues in TyrH without seeing definitive effects on enzyme activity (*27*). Still, site-directed mutagenesis of conserved histidine residues has established histidines 331 and 336[2] as ligands to the iron in both TyrH and PheH (*11*, *12*). Using an alternative approach involving anti-idiotype antibodies, Jennings et al. identified residues 263−289 of PheH (309−335 of TyrH) as a possible region for pterin binding (*28*).

With the availability of the three-dimensional structures of the catalytic domains of TyrH and PheH (*9*, *10*), it is possible to identify residues which are located in the active site of these enzymes. The active sites of the two enzymes are effectively identical. Both are hydrophobic clefts framed by four helices with the iron near the bottom. Many of the residues lining the active site are hydrophobic, consistent with the hydrophobicity of the substrates. In the work described herein, we have focused on active site residues which are conserved among all three hydroxylases and are likely to be charged at pH 7.1. The residues meeting these criteria in TyrH are arginine 316, glutamate 326, aspartate 328, histidine 331, glutamate 332, histidine 336, and glutamate 376. Histidines 331 and 336 and glutamate 376 are ligands to the active site iron. The effects of mutagenesis of these two histidine residues have been described previously for TyrH and PheH. In both cases, the mutant proteins are inactive (*29*, *30*). Mutagenesis of the remaining iron ligand glutamate 376 would presumably have a similar effect and was consequently not carried out in the experiments described here. The other four charged residues have now been altered by site-directed mutagenesis and the resulting proteins characterized.

The kinetic mechanism depicted in Scheme 2 provides a framework for the interpretation of the effects of the mutations described here. Steady-state kinetic analyses to date are consistent with both the amino acid and the tetrahydropterin substrate having low commitments to catalysis[3] (*20*, *24*). Consequently, the K_M values for both of these substrate are reasonable approximations of the dissociation constants. For the mechanism depicted in Scheme 2, the V/K_{tyr} value includes the rate constants for both the binding of tyrosine and the formation of the hydroxylating intermediate. This conclusion is supported by the ^{18}O kinetic isotope effects measured by Francisco et al. (*24*). Because the formation of the hydroxylating intermediate is expected to be irreversible, the rate of the subsequent transfer of oxygen to the amino acid is not reflected in the V/K_{tyr} value. The V_{max} value for dihydropterin formation reflects the rates

[2] The numbering of residues is that of rat tyrosine hydroxylase. Rat phenylalanine hydroxylase is 46 residues shorter due to the smaller size of the regulatory domain in that enzyme. Thus, the homologous residues in PheH would differ in numbering by 46 from those used here.

[3] P. F. Fitzpatrick, unpublished observations.

both for the formation and for the further reaction of the hydroxylating intermediate. With the wild-type enzyme, formation of the hydroxylating intermediate is the slow step, so the V_{max} value primarily reflects this step. The relative rates of the two pathways by which the hydroxylation intermediate can react further can be determined by comparison of the rates of formation of DOPA and dihydropterin (*26*, *31*).

Clearly, arginine 316 is critical to TyrH activity. Mutagenesis of arginine 316 has the most dramatic effect of any of the mutations described here on the properties of TyrH. Substitution with uncharged alanine, serine, or glutamine residues resulted in an insoluble protein. It was only upon substitution with a positively charged lysine residue that it was possible to obtain a soluble enzyme. Even this relatively conservative mutation resulted in a mutant protein with very low activity. Although the K_{6-MPH_4} value is only an apparent value because it could not be measured at a saturating tyrosine concentration, it appears to be only slightly affected by the substitution of lysine for arginine 316. In contrast, the K_{tyr} value for R316KTyrH is at least 400-fold greater than the wild-type value; we can only place a lower limit on this value because of the limited solubility of tyrosine. On the basis of the linear dependence on the tyrosine concentration of the initial velocity, the K_{tyr} value is greater than 10 mM. We could determine the V/K_{tyr} value from the dependence of the initial rate on the tyrosine concentration; the value is 3700-fold lower than the value for wild-type TyrH. This decrease could be entirely due to a decrease in the extent of binding of tyrosine, or it could also reflect a decrease of up to 10-fold on the rate of formation of the hydroxylating intermediate. Since we could not achieve saturation by tyrosine, we could not measure V_{max} values for formation of either product. However, the ratio of DOPA production to dihydropterin production could be measured, since it is independent of the concentrations of the substrates. Transfer of the oxygen to the amino acid is still relatively competitive with the unproductive breakdown of the hydroxylating intermediate, although there is an increase in the amount of unproductive turnover. This suggests that the lesion in this protein lies before the formation of the hydroxylating intermediate, and that the hydroxylating intermediate reacts relatively normally if it can form. A minimalist interpretation of the data is that arginine 316 is required for productive binding of tyrosine. A reasonable basis for this is that the guanidino moiety of arginine 316 binds the carboxylate moiety of tyrosine. No structure is available for tyrosine hydroxylase with an amino acid bound. In the available structure with no substrates bound, the guanidino nitrogens of arginine 316 are 9.7 and 11.6 Å from the iron atom. This distance would allow a tyrosine to bind with its carboxylate interacting with the arginine and the aromatic ring appropriately positioned for hydroxylation by an iron−oxo species.

In contrast to the dramatic effects seen upon mutagenesis of arginine 316, mutagenesis of glutamate 326 to alanine had only minor effects on the catalytic properties of TyrH. The finding that E326ATyrH is an active tyrosine hydroxylase demonstrates that glutamate 326 is not essential for activity. Still, the mutation did have some effects on catalysis. The K_{tyr} and K_{6-MPH_4} values are comparable between E326ATyrH and wild-type TyrH, suggesting that binding of substrates is unaffected and consequently any binding interactions between substrates and glutamate 326 are weak. Both the V/K_{tyr} value and the V_{max} for dihydropterin formation are decreased about 3-fold, consistent with a decrease of this magnitude in the rate of formation of the hydroxylating intermediate. Consistent with a change in the properties of the hydroxylating intermediate in the mutant protein, there is also an increase in the amount of unproductive formation of dihydropterin. Thus, substitution of alanine in position 326 affects the chemical steps in hydroxylation rather than binding. A reasonable explanation for these results is that the mutation alters the overall conformation of the active site.

Mutagenesis of aspartate 328 to alanine did not produce soluble protein in our hands, but substitution with either asparagine or serine resulted in a small quantity of soluble mutant protein. The levels were a little higher for D328STyrH, so it was purified for study. D328STyrH is deficient in tyrosine binding, with a 26-fold increased K_{tyr} value. The V_{max} value for dihydropterin formation is decreased about 8-fold. Thus, once tyrosine binds, the hydroxylating intermediate can form at a reduced rate. The decrease in the V/K_{tyr} value of 200-fold reflects the decrease in both the level of tyrosine binding and the rate of formation of the hydroxylating intermediate. Once formed, the hydroxylating intermediate is quite capable of forming DOPA; each time the hydroxylating intermediate is formed there is a 60% chance the oxygen will be transferred to tyrosine. These data suggest that the carboxylate moiety of aspartate 328 binds the amino moiety of tyrosine. The carboxylate oxygens of aspartate 328 are 8.8 and 11 Å from the iron atom. This would allow the amino moiety of the substrate to form a salt bridge with aspartate 328 while the aromatic ring would be properly positioned for hydroxylation and the carboxylate moiety would interact with the nearby arginine 316.

The final residue which was mutated to alanine was glutamate 332. In this instance, tyrosine binding is unimpaired, but the K_{6-MPH_4} value is 10-fold higher than for the wild-type enzyme. There is a much greater effect on the V_{max} value for DOPA formation than on that for dihydropterin formation. This result is best explained by expanding the minimal kinetic mechanism of Scheme 2 and considering the individual chemical steps involved. Heavy atom kinetic isotope effects with ^{18}O are consistent with the initial step in the formation of the hydroxylating intermediate being an irreversible reaction between oxygen and the tetrahydropterin (*24*). The relatively small effect of the E332A mutation on the rate of dihydropterin formation suggests that this initial reaction with oxygen is relatively unimpaired in the mutant protein. Instead, a subsequent step required for amino acid hydroxylation is perturbed. The identity of the hydroxylating intermediate in the pterin-dependent hydroxylases has not been established. A mechanism consistent with the available data and previous mechanistic proposals is that the initial reaction of oxygen and tetrahydropterin forms a peroxypterin (*32*). This would be followed by heterolytic cleavage of the peroxide O−O bond to form the hydroxypterin product and a high-valence iron−oxo species, the actual hydroxylating intermediate (*24*, *33*−*35*). Such a mechanism is fully consistent with the effects of this mutation. Removal of the carboxylate of glutamate 332 would result in slightly altered binding of tetrahydropterin. The reaction between oxygen

and tetrahydropterin could still occur, but the interaction of the terminal oxygen of the peroxypterin with the iron would be disturbed. This would greatly decrease the rate of oxygen transfer to the iron, while allowing the unproductive breakdown of the peroxypterin to still occur. Thus, the effects of this mutation on both binding and catalysis are consistent with altered binding of tetrahydropterin in the mutant protein.

Dickson et al. (*5*) have described the effects of altering the residue in PheH corresponding to glutamate 332 to alanine. They reported that the K_M value for tetrahydropterin was increased some 70-fold, while the rate of phenylalanine hydroxylation was decreased 200-fold. The effect on the rate of dihydropterin production was not determined in that case. The results presented here with TyrH agree qualitatively with these results.

Goodwill et al. have recently described the structure of the catalytic domain of rat tyrosine hydroxylase with dihydrobiopterin bound (*36*). The side chain of the pterin is 4 Å from the carboxylate of glutamate 332, too far for a direct interaction. In the structure of the catalytic domain of human phenylalanine hydroxylase, the corresponding glutamate residue forms a hydrogen bond with a water ligand to the iron (*10*). Thus, the available structural data suggest that any interaction between glutamate 332 and the pterin is indirect. However, it must be kept in mind that the TyrH structure was determined with dihydropterin instead of tetrahydropterin. In addition, a conformational change must occur after tyrosine binds, so the available structures may not reflect accurately the final positions of these residues.

The results of the studies described here indicate that all of the conserved charged residues located in the active site of TyrH contribute to catalysis to some extent. Arginine 316 and aspartate 328 are critical for binding of tyrosine, while glutamate 332 is required for tight binding of tetrahydropterin. The relatively minor effects of mutating glutamate 326 suggest that it is required to maintain the active site structure. The present results also provide strong support for the kinetic mechanism depicted in Scheme 2, in which the rate-limiting step is the formation of a highly reactive hydroxylating intermediate.

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