Expression and Characterization of the Catalytic Core of Tryptophan Hydroxylase

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Wild type rabbit tryptophan hydroxylase (TRH) and two truncated mutant proteins have been expressed in Escherichia coli. The wild type protein was only expressed at low levels, whereas the mutant protein lacking the 101 amino-terminal regulatory domain was predominantly found in inclusion bodies. The protein that also lacked the carboxyl-terminal 28 amino acids, TRH102–416, was expressed as 30% of total cell protein. Analytical ultracentrifugation showed that TRH102–416 was predominantly a monomer in solution. The enzyme exhibited an absolute requirement for iron (ferrous or ferric) for activity and did not turn over in the presence of cobalt or copper. With either phenylalanine or tryptophan as substrate, stoichiometric formation of the 4α-hydroxypterin was found. Steady state kinetic parameters were determined with both of these amino acids using both tetrahydrobiopterin and 6-methyltetrahydropterin.

Tryptophan hydroxylase (TRH1, EC 1.14.16.4) carries out the 5-hydroxylation of tryptophan via the oxidation of tetrahydropterin and the reductive incorporation of molecular oxygen (Scheme 1). In mammalian metabolism the reaction catalyzed by TRH precedes α-decarboxylation and is believed to be the initial and rate-limiting process in the production of the neurotransmitter serotonin (5-hydroxytryptamine). Although TRH has been studied since the early 70s, enzymological characterization has been impeded by the limited quantity of active enzyme available from native or heterologous sources, the exceedingly low specific activity of the isolated enzyme, and the rapid decrease in activity observed during purification or storage (1–9).

TRH is a member of the small family of pterin-dependent aromatic amino acid hydroxylases that includes tyrosine hydroxylase (TYH) and phenylalanine hydroxylase (PAH). Each of these enzymes catalyzes the addition of an oxygen atom to the ring of an aromatic amino acid substrate. The bulk of what is currently known of the reaction mechanism of these enzymes has come from studies of the latter two (10). Both PAH and TYH require ferrous iron for activity (11, 12); however, the exact role for the iron in catalysis is undefined. The primary structures of these enzymes are known from a variety of organisms. Sequence comparisons and deletion mutageneses have identified three functional regions: an amino-terminal regulatory domain, a catalytic domain, and a carboxyl-terminal interface (13–17). The regulatory domains of the three hydroxylases show no similarities, whereas the catalytic domains are homologous, with sequence identities of 32–75%. Enzymes lacking the regulatory domain are catalytically active (13, 14, 17). The carboxyl-terminal 24 amino acids of TYH form a long helix demonstrated to be responsible for the tetrameric structure of the enzyme (13, 18); this helix is presumed to have a corresponding function in TRH and PAH.

We report here the purification and preliminary characterization of a mutant protein containing only the catalytic core of TRH. The rationale for the truncations was to increase the heterologous expression and/or stability of the enzyme by removing both the regulatory and interface domains. This doubly truncated form of the enzyme serves as the first viable model enzyme for detailed mechanistic studies of the catalytic reaction mechanism of TRH. The high specific activity of this enzyme has allowed the analysis of several fundamental properties of this important enzyme, including catalytic specificity and metal dependence.

EXPERIMENTAL PROCEDURES

Materials—Tryptophan, 5-hydroxytryptophan, and β-mercaptoethanol were obtained from Sigma. 6-Methyltetrahydropterin was synthesized according to Fitzpatrick (19). Tetrahydrobiopterin was purchased from Calbiochem. MES and dithiothreitol were purchased from Research Organics, Inc. Catalase was purchased from Boehringer Mannheim. Ceramic hydroxyapatite was from Bio-Rad, and Q-Sepharose was obtained from Amersham Pharmacia Biotech. Isopropyl-β-thiogalactoside was from United States Biochemical Corp. Cuprous chloride, cupric sulfate, ferric sulfate, and ferrous ammonium sulfate were from Sigma. Cobalt chloride was purchased from Mallinkrodt. Agarose (SeaKem) was from FMC. [3,5-3H]Tyrosine was from Amersham Pharmacia Biotech. Ultima polymerase and deoxynucleotides for polymerase chain
reaction were obtained from Perkin-Elmer. Plasmid pET3d and *Escherichia coli* BL21 (DE) were obtained from Novagen. *E. coli* CJ236 was from Invitrogen. Plasmid pTZ18R was from Amersham Pharmacia Biotech. Oligonucleotides were custom-synthesized using an Applied Biosystems model 380B synthesizer. Restriction and DNA modification enzymes were purchased from New England Biolabs. pET3d was purified using the Qiagen midi-prep plasmid preparation kit.

**Vectors for TRH Expression**—The construct for expression of wild type TRH was made by polymerase chain reaction subcloning from the rabbit cDNA-derived plasmid prTRH479 (20). *NcoI* and *BamHI* restriction sites were incorporated at the 3' and 5' ends of the gene, respectively, via non-complementary oligonucleotide tails. The 1.3-kilobase pair product was then digested and subcloned into pET3d and pTZ18R to form the wild type TRH constructs pEWOH2 and pWH1 for expression and mutagenesis, respectively. In pEWOH2 the start codon for the TRH gene is 6 bases from its ribosome binding site and 55 bases from the T7 promoter.

Deletion of the amino-terminal 101 amino acids of TRH was achieved using the Bio-Rad adaptation of the methods of Kunkel et al. (21) with single-stranded uracil-containing DNA derived from pWH1. The oligonucleotide 5' ATGAAAGGAAGGAGCCATGAGGTCTCTTGGTTCACCA 3' was used to incorporate a NcoI restriction site (in bold) into the TRH gene adjacent to position 101. The resulting plasmid was digested with NcoI and *BamHI* and ligated into pET3d to obtain pER880, a truncated construct. In a similar manner, the relationship of pEWOH101 was unchanged from that in pEWOH2.

Exclusion of the carboxyl-terminal interface helix from translation was achieved using a variation of the Stratagene quick change mutagenesis method. pEWOH101 was used as a template for a polymerase chain reaction reaction in which two complementary oligonucleotides (5' GCAAGCTGTTACAGTGGTCAAAAAGGCTGCGC 3' and 5' GGACGCTGTTAGCCATGTACGTTGGTTCACC 3') were used to mutate codons Ile417 and Met421 to stop codons (in bold). The resulting transformed BL21 (DE) cells were screened for expression of the doubly truncated protein using SDS-polyacrylamide gel electrophoresis. The mutation was confirmed by sequencing the entire gene of a plasmid from a cell line that expressed active TRH102–416; this was designated pEWOH101SH.

**Protein Expression and Purification**—Aliquots from frozen cell stocks were plated (240 µl/liter of culture) on LB agar (100 µg/ml carbenicillin). After 9 h at 37 °C, the cells from two plates were resuspended in 10 ml of LB broth and used to inoculate 1 liter of LB broth (100 µg/ml ampicillin). The culture was grown with vigorous shaking at 37 °C for 10 min, and the supernatant from 1 liter of cell culture was 10 mg with a specific activity of 0.6 µmol of hydroxytryptophan produced per min/mg.

**Ultra-centrifugation**—Sedimentation equilibrium analyses were carried out at 10 °C in a Beckman model XLA ultracentrifuge. Concentrated TRH102–416 was diluted to 5, 10, or 15 µM in 200 mM (NH₄)₂SO₄, 100 mM MES, pH 7.2. The system was assessed as having attained equilibrium when scans at 280 nm taken at 2-h intervals were identical. The data used for analysis were averages of 20 successive scans. The absorbance values as a function of radial position were fit using Kaleidagraph software to either Equation 1, which describes the sedimentation of a monomer, or Equation 2, which describes the sedimentation of a self-associating species. The terms used in Equations 1–3 are as follows: N, the stoichiometry of an enzyme monomer; M, the molecular weight of an enzyme monomer; nMo, the absorbance at the reference radius r0; Kc, the association constant; C, the base-line offset; ω, the partial specific volume (calculated to be 0.723 from the amino acid content of TRH102–416; p); the buffer density; and ω₂, the angular velocity in radians/s. The concentration of protein was determined using an ε₂₉₀ value of 35.2 mM⁻¹ cm⁻¹ calculated by the method of Pace et al. (22).

\[
A_0 = \exp(lnA_0 + HM(r^2 - r_0^2)) + C
\]  
(Eq. 1)  
\[
A_r = \exp(lnA_0 + HM(r^2 - r_0^2)) + \exp(lnA_0 + ln(Kc)HNMM(r^2 - r_0^2)) + C
\]  
(Eq. 2)  
\[
H = \frac{(1 - 6\rho_0)^2}{2\pi^2}
\]  
(Eq. 3)

**Enzyme Assay**—An HPLC-based assay for enzyme activity was used for samples during purification. The 500-µl reaction mixture contained enzyme, 0.2 mg/ml catalase, 200 µM tryptophan, 200 µM 6-MePH, 100 µM ferrous ammonium sulfate, 15 mM β-mercaptoethanol, 100 mM MES, pH 7.0. 275 nm and an emission of 375 nm. Under these conditions 5-hydroxytryptophan and tryptophan were detected using a Waters 470 fluorescence detector with an excitation λ of 290 nm and an emission λ of 340 nm. Under those conditions 5-hydroxytryptophan and tryptophan had retention times of 1.7 and 3.9 min, respectively. When phenylalanine was used as a substrate, tyrosine was detected with an absorption of 275 nm/abs 320 nm wavelength cut-off filter perpendicular to the light source was used. The concentration of tryptophan was monitored independently of the substrate or the formation of the fluorescent 7,8-dihydropyridine. The amount of 5-hydroxytryptophan generated was quantified by comparing the fluorescence yield to that of 5-hydroxytryptophan standards measured on the same instrument in the presence of comparable quantities of other substrates. The concentration dependence data were fit to Equations 4 and 5. Equation 5 was

\[
A_r = \exp(lnA_0 + HM(r^2 - r_0^2)) + \exp(lnA_0 + ln(Kc)HNMM(r^2 - r_0^2)) + C
\]  
(Eq. 2)  
\[
H = \frac{(1 - 6\rho_0)^2}{2\pi^2}
\]  
(Eq. 3)
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used when substrate inhibition was observed; \( K_m \) is the inhibition constant for the substrate.

\[
u = \frac{V A}{K_m + A} \quad \text{(Eq. 4)}
\]

\[
u = \frac{V A}{K_m + A + A^2/K_m} \quad \text{(Eq. 5)}
\]

The metal requirement of TRH_{102–416} was determined using sequential stopped-flow spectrophotometry. The apoenzyme (10 \( \mu \)M, 2.5 \( \mu \)M final concentration) in 100 mM MES, 200 mM \((NH_4)_2SO_4\), pH 7.0, was first mixed with 6-MePH (400 \( \mu \)M, 100 \( \mu \)M final concentration) and tryptophan (200 \( \mu \)M, 50 \( \mu \)M final concentration). After 1 s this was mixed with another solution containing 100 mM metal ion (50 \( \mu \)M final concentration). The second mixing step initiated data collection.

A modified version of the assay of Shiman et al. (23) was used to measure tyrosine formation from phenylalanine. Enzyme (typically 2–10 \( \mu \)M) in air-saturated 100 mM MES, 200 mM \((NH_4)_2SO_4\), 100 mM ferrous ammonium sulfate, 25 \( \mu \)g/ml catalase, pH 7.0, with varied concentrations of 3,5-(\( \alpha \)-H)tyrosine and tetrahydropterin. The reaction was carried out for 1 min at 15 °C.

Pterin Products and Stoichiometry—Enzyme (10 \( \mu \)M final) was mixed with tryptophan (200 \( \mu \)M), phenylalanine (200 \( \mu \)M), or tyrosine (650 \( \mu \)M) in 100 mM MES, pH 7.2, 200 mM \((NH_4)_2SO_4\), 100 mM ferrous ammonium sulfate, at 15 °C, in a 4-mm path length quartz cuvette. After recording a base line with this solution, the reaction was initiated by the addition of either 6-MePH, or BH, to 100 \( \mu \)M, and spectra were recorded every 5 s for 400 s. The amount of hydroxylated amino acid produced was then determined by HPLC. The spectra generated during the reaction were analyzed globally using the program Specfit (Spectrum Software Associates) to determine the spectra of the pterin products.

Iron Determination—The iron content of the enzyme was determined by atomic absorption spectroscopy, using a slight variation of the method of Ramsey et al. (24). Samples were dialyzed into 100 mM MES, 200 mM \((NH_4)_2SO_4\), pH 7.0, and then diluted 5-fold in 2.5 mM nitric acid. After 30 min on ice the samples were diluted 10-fold with water and centrifuged at 12,000 \( \times \) g for 10 min. Aliquots of the supernatant were analyzed for iron using a Perkin-Elmer model 2380 atomic absorption spectrophotometer equipped with a graphite furnace.

**RESULTS**

Expression—The T7 polymerase-based pET expression system of Studier (25) was used to express wild type TRH and two truncated mutant proteins. The wild type TRH was expressed as less than 1% of the total cell protein. This level of expression was unaffected by temperature over the range 20–37 °C (data not shown). At 20 °C, the TRH activity reached a maximum level of approximately 4 nmol/min/ml culture 7 h after induction (Fig. 1A). All of the wild type TRH was soluble. In contrast, the protein lacking the amino-terminal regulatory domain, TRH_{102–444}, was expressed at much higher levels. Based upon SDS-polyacrylamide gel electrophoresis of cell lysates after induction, TRH_{102–444} was expressed as 30–35% of the total cell protein under all conditions tested. However, when cells were grown at 37 °C, 95% of the TRH_{102–444} was found in inclusion bodies. The fraction of soluble TRH_{102–444} could be increased by decreasing the growth temperature to 17 °C, but the greatest activity found under any condition with this protein was only 40% that observed with the wild type protein (see Fig. 1A). Moreover, during attempts to purify TRH_{102–444}, the enzyme showed a pronounced tendency to lose activity and precipitate.

Although the absolute levels of expression seen with TRH_{102–444} were sufficient, the insolubility of the protein and the low activity suggested that it was not folding properly. TRH is believed to be held together as tetramer by hydrophobic helices at the carboxyl termini of each monomer (6, 8, 16, 27), similar to TYH (13, 18). Given the tetrameric nature of TRH, it was considered possible that the presence of even a single unfolded subunit within the tetramer would be sufficient to render the entire tetramer unstable. Since the interface helix is not required for activity in these hydroxylases (13, 15, 27), a mutant protein lacking both the regulatory domain and the carboxyl-terminal helix was examined. This form of TRH, TRH_{102–416}, was expressed at a level similar to that observed with TRH_{102–444}. However, based on SDS-polyacrylamide gel electrophoresis and enzyme activity, 80% of the protein was soluble, so that the active, soluble TRH_{102–416} was 25–30% of the total cell protein. The amount of enzyme activity produced as TRH_{102–416} was approximately 10 times that observed with the wild type enzyme and 25 times that obtained with TRH_{102–444} (Fig. 1A). Consequently, TRH_{102–416} was selected for further characterization.

Purification—TRH_{102–416} could be purified in three steps, a Q-Sepharose column, ammonium sulfate fractionation, and a hydroxyapatite column (Fig. 1B). TRH_{102–416} showed marked instability at low ionic strength. Maintaining the enzyme in a
minimum of 100 mM (NH₄)₂SO₄ during purification greatly enhanced recovery from each chromatographic step. The enzyme stability was further enhanced by ferrous iron and dithiothreitol. The enzyme could be stored at −70 °C indefinitely without loss of activity. Because of the improved stability which resulted, the enzyme was typically stored in the presence of 100 µM ferrous ammonium sulfate. If ferrous ammonium sulfate was omitted from purification buffers, the resulting enzyme contained no detectable iron (<0.05 atom/monomer).

**Ultracentrifugation**—Since TRH 102–416 no longer contains the helix that is proposed to be necessary for oligomerization, the mutant protein should be a monomer. Equilibrium ultracentrifugation was used to analyze its quaternary structure. Data were collected using initial protein concentrations of 5–15 mM and rotor speeds of 16,000, 19,000, and 22,000 rpm. When the data were analyzed assuming that a single monomeric species was present, the average molecular weight over all conditions was 42,400 ± 4381, compared with a molecular weight of 36,319 calculated from the DNA sequence. These results suggested that species larger than a monomer were present. To examine this possibility, the data were fit to models describing monomer-dimer, monomer-trimer, monomer-tetramer, monomer-hexamer, and monomer-octamer equilibria. In each case the molecular weight was fixed at 36,319. The data were best fit using a monomer-tetramer model (Table I). No improvements in the quality of the fits were seen if species larger than a tetramer were considered. The average $K_a$ value obtained by fitting each data set to a model describing a monomer-tetramer equilibrium was 1.33 × 10⁻¹³ M⁻³. Fig. 2 shows representative fits to this model of data obtained at three rotor speeds of 16,000, 19,000, and 22,000 rpm.

**Hydroxypyrin Formation**—With tyrosine hydroxylase and phenylalanine hydroxylase, the initial pterin product of catalysis is a 4a-hydroxypyrin (28–30). Although it is assumed that this is also a product with tryptophan hydroxylase, as shown in Scheme 1, formation of a hydroxypyrin by tryptophan hydroxylase has not been demonstrated directly. Although hydroxypyrins are not stable in solution for extended periods, they can be observed spectrally if the enzyme concentration is high enough to rapidly generate micromolar levels prior to hydrolysis. Consequently, high concentrations of TRH 102–416 (10 µM) were used to consume 100 µM tetrahydrobiopterin in the presence of excess tryptophan or phenylalanine. Near ultraviolet absorbance spectra of the reaction were collected every 5 s using a diode array spectrophotometer. The formation of the hydroxypyrin was clearly detectable at 246 nm, where its absorbance is maximal, when either tryptophan or phenylalanine was the amino acid substrate (Fig. 3). After the spectral changes were complete, the amount of hydroxylated amino acid produced was determined by HPLC. With tryptophan as substrate 103 nmol of hydroxytryptophan were produced after complete oxidation of 100 nmol of tetrahydrobiopterin. Similarly, oxidation of 100 nmol of tetrahydrobiopterin in the presence of phenylalanine produced 99 nmol of tyrosine. Similar results were obtained with 6-MePH₄ (Table II). However when tyrosine was used as a substrate only 1.2 nmol of 3,4-dihydroxyphenylalanine were produced upon the oxidation of 100 nmol of tetrahydrobiopterin.

The sequential spectra collected during turnover were fit globally to the model in Scheme 2 to determine the spectra of the individual pterin species produced. The data were well fit by such a model (results not shown). The spectrum of the 4a-hydroxybipterin determined by this method agreed well with previously published spectra (31), with an $e_{426}$ value of 18.1 M⁻¹ cm⁻¹ (Fig. 3). The spectra of the subsequently formed pterin species agreed with previously described spectra of the quinonoid dihydrobiopterin and 7,8-dihydrobiopterin (31, 32).

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**Table I**

| Speed [rpm] | [TRH 102-416] | Monomer | Monomer-dimer | Monomer-tetramer | Monomer-hexamer | Monomer-octamer |
|------------|----------------|---------|---------------|------------------|-----------------|-----------------|
| 16,000     | 5              | 0.0077* | 0.0066*       | 0.0049           | 0.0043          | 0.0042          |
| 16,000     | 10             | 0.085   | 0.078         | 0.065            | 0.062           | 0.063           |
| 16,000     | 15             | 0.077   | 0.069         | 0.060            | 0.060           | 0.064           |
| 19,000     | 5              | 0.012   | 0.011         | 0.0097           | 0.0085          | 0.0079          |
| 19,000     | 10             | 0.14    | 0.12          | 0.095            | 0.094           | 0.099           |
| 19,000     | 15             | 0.10    | 0.085         | 0.068            | 0.073           | 0.081           |
| 22,000     | 5              | 0.013   | 0.014         | 0.013            | 0.012           | 0.011           |
| 22,000     | 10             | 0.10    | 0.088         | 0.079            | 0.081           | 0.088           |
| 22,000     | 15             | 0.088   | 0.084         | 0.059            | 0.064           | 0.069           |

* Initial concentration of protein.

**Table II**

| Speed [rpm] | $K_a$ [M⁻³] | $e_{426}$ [M⁻¹ cm⁻¹] |
|------------|------------|---------------------|
| 16,000     | 0.0077     | 18.1                |
| 16,000     | 0.0066     | 18.1                |
| 16,000     | 0.0049     | 18.1                |
| 16,000     | 0.0043     | 18.1                |
| 16,000     | 0.0042     | 18.1                |

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**Fig. 2** Analytical ultracentrifugation of TRH 102–416 at 22,000 rpm. The initial concentrations of enzyme before centrifugation began were 5 µM (□–□), 10 µM (□ – □), or 15 µM (□ – □). The lines represent fits of the data to a monomer-tetramer equilibrium with a $K_a$ value of 1.3 × 10⁻¹³ M⁻³ using Equation 2.
Steady State Kinetic Analyses for TRH 102–416—Continuous assays were developed as a consequence of the substantial increase in activity of TRH 102–416 compared with TRH from other sources. The assay for hydroxylation of tryptophan followed the increased fluorescence of the product at wavelengths greater than 320 nm. Selectively exciting the reaction at 300 nm minimized the emission contributions from the substrate and fluorescent pterin products. With the alternate substrate phenylalanine, the accumulation of tyrosine could be observed as an absorbance increase at 275 nm.

Tryptophan, phenylalanine, and tyrosine were examined as substrates for TRH 102–416. Both tryptophan and phenylalanine were hydroxylated efficiently, producing exclusively 5-hydroxytryptophan and 4-hydroxyphenylalanine, respectively. Under the experimental conditions, no production from phenylalanine of 3-hydroxyphenylalanine or 3,4-dihydroxyphenylalanine could be detected by HPLC. Very small quantities of 3,4-dihydroxyphenylalanine formed from tyrosine could be detected. The hydroxylation rate of tyrosine by TRH 102–416 under the conditions of the experiment was at least 5000-fold slower than that observed with tryptophan and phenylalanine. For this reason steady state kinetic parameters were determined only with tryptophan and phenylalanine using 6-MePH₄ and BH₄. Significant substrate inhibition was seen when either amino acid was varied at a fixed concentration of either BH₄ or 6-MePH₄ (Fig. 4). Substrate inhibition was also seen when either tetrahydropterin was varied with tryptophan, but little or no inhibition was seen with either tetrahydropterin when phenylalanine was the fixed substrate (Fig. 4). Table III summarizes the kinetic parameters.

The Metal Requirement of TRH 102–416—Both tyrosine hydroxylase and phenylalanine hydroxylase have been shown to be iron-containing enzymes (33–35), so that tryptophan hydroxylase is also assumed to require iron for activity. If no iron was included in the buffer when purifying TRH 102–416, the resulting protein contained no significant iron. Apoenzyme prepared in this fashion was used to determine the metal requirement of tryptophan hydroxylase. The assays were done at high (2 mM) enzyme concentrations to avoid ambiguities due to the presence of contaminating metals. The apoenzyme was mixed with tryptophan and 6-MePH₄ and then 1 s later with individual metals at a final concentration of 50 μM. The formation of hydroxytryptophan was followed by fluorescence. Representative kinetic traces are shown in Fig. 5. No activity was observed in the absence of added metal. In the presence of 50 μM ferrous iron there was a constant rate of product formation beginning almost immediately after mixing. The same rate was seen with 10 μM ferrous iron (results not shown). In contrast, in the presence of an equal concentration of ferric iron, a significant lag was seen before the activity reached the same level as was...
seen with ferrous iron. The enzyme was not active with copper or cobalt.

DISCUSSION

The three tetrahydropterin-dependent hydroxylases, TYH, PAH, and TRH, constitute a small family of proteins that catalyze the hydroxylation of aromatic amino acids. Because of the availability of PAH from liver, studies of this enzyme have the most extensive history (36). More recently, the availability of recombinant TYH has resulted in a much greater understanding of that enzyme (10). In contrast, understanding the structure and mechanism of tryptophan hydroxylase has made little progress. Natural sources such as brain have proved to contain too little enzyme for useful purification, resulting in very low amounts of protein with low specific activities (1, 2, 4). A number of groups have described preparations of TRH from recombinant sources, but these typically involved enzyme of low or indeterminate specific activity (16, 37, 38). The limited amounts of material available have severely restricted analyses of the mechanism of this important enzyme.

Sequence comparisons of all three hydroxylases routinely show that the carboxyl-terminal 340 amino acids form a homologous region, whereas the amino-terminal sequences diverge widely (20). Deletion mutageneses of all three enzymes have defined the catalytic core which contains the residues required for catalysis. PAH lacking the amino-terminal 141 amino acid residues and the carboxyl-terminal 43 residues is reported to retain activity (15). Similar results have been reported for TYH (13, 14), whereas TRH is reported to retain some activity if as many as 106 residues are deleted from the amino terminus and as many as 19 residues are deleted from the carboxyl terminus (9, 39). Based upon such results, the amino-terminal portions of these proteins are generally accepted to be regulatory domains, containing phosphorylation sites and required for allosteric properties (37, 40–42). The 42 carboxyl-terminal residues of each monomer are responsible for tetramer formation, primarily due to the presence of a 24-residue helix at the end (13, 18). The remaining 300 residues form the catalytic domains of each hydroxylase. Removal of the regulatory domains from either TYH or PAH has only subtle effects on the substrate specificities or catalytic rates (17).
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TABLE III

Steady state kinetic parameters for TRH102–416

| Varied substrate | Fixed substrate | Equation fit | V_{max} \text{ min}^{-1} | K_m \mu M | V/K \text{ min}^{-1} \mu M | K_{cat} \mu M |
|------------------|-----------------|--------------|---------------------------|-----------|-----------------------------|-------------|
| Tryptophan       | BH_4             | 5            | 50.4 ± 2.8                | 47.9 ± 4.2 | 1.05 ± 0.04                 | 146 ± 14    |
| Phenylalanine    | BH_4             | 5            | 92.0 ± 4.1                | 60.6 ± 5.2 | 1.51 ± 0.07                 | 916 ± 110   |
| Tryptophan       | 6-MePH_4         | 5            | 24.0 ± 0.3                | 5.8 ± 0.23 | 4.12 ± 0.12                 | 966 ± 88    |
| Phenylalanine    | 6-MePH_4         | 5            | 97 ± 6.1                  | 102 ± 9.8  | 0.94 ± 0.04                 | 228 ± 23    |
| BH_4             | Tryptophan*      | 5            | 50.5 ± 0.65               | 135 ± 16   | 0.37 ± 0.01                 | 206 ± 30    |
| 6-MePH_4         | Tryptophan*      | 5            | 68.3 ± 7.9                | 177 ± 25   | 0.40 ± 0.01                 | 71.5 ± 11   |
| BH_4             | Phenylalanine#   | 4            | 64.7 ± 0.65               | 14.2 ± 0.51| 4.56 ± 0.14                 | 2540 ± 446  |
| 6-MePH_4         | Phenylalanine#   | 5            | 31.6 ± 0.56               | 14.6 ± 0.8 | 2.16 ± 0.086                | 2540 ± 446  |

*a* Determined at 150 μM BH_4.
*b* Determined at 100 μM 6-MePH_4.
*c* Determined at 200 μM tryptophan.
*d* Determined at 100 μM phenylalanine.

FIG. 5. The metal requirement of TRH102–416. Traces show the fluorescence changes due to 5-hydroxytryptophan formation upon mixing apo-TRH102–416 with the indicated metals at a concentration of 50 μM in the presence of 6-MePH_4 and tryptophan at 15 °C.

In our hands, expression of the wild type rabbit TRH resulted in low levels of expression, consistent with the observations of others (38). Although removal of the regulatory domain resulted in a significant increase in the level of expression of TRH102–444, this form of the protein was only slightly soluble. It was only upon removal of the long helix in the tetramer interface that high levels of soluble active enzyme were obtained. A possible reason for this increase in solubility of TRH102–416 is that tetramers of TRH102–444 contain mixtures of correctly folded and incorrectly folded monomers. Any improperly folded subunit may render an entire tetramer unstable. Even if not all of the monomeric TRH102–416 is correctly folded, any improperly folded subunits would not be expected to affect the stability of other monomers. Irrespective of whether this is the correct reason for its increased solubility, TRH102–416 is expressed at sufficiently high levels for mechanistic studies.

Wild type TRH is a tetramer (1, 4). In contrast, TRH102–416 is monomeric at and above concentrations typically encountered in kinetic experiments and only forms oligomers at a relatively high concentration. This is the result expected upon removal of the intersubunit helix. There are clearly still some interactions among the monomers in TRH102–416 despite the lack of this helix. The structure of TYH shows that the tetramerization domain contains the carboxyl 42 residues, which include the intersubunit helix (18). In addition, there are other interactions across dimer interfaces. Similar interactions in TRH are presumably the reason for the weak formation of tetramers.

TRH102–416 clearly requires ferrous iron for activity, as do both TYH and PAH (11, 12, 43). Although TRH102–416 is active with ferric iron, there is a significant lag in formation of hydroxytryptophan in the presence of this metal. Both TYH and PAH are routinely found to have the active site iron in the ferric form when purified (44, 45). The iron must be reduced to the ferrous form for catalysis; tetrahydrobiopterin appears to be the physiological reductant (11, 24, 43). The lag seen in the formation of hydroxytryptophan by TRH102–416 in the presence of ferric iron is consistent with a similar phenomenon occurring with this protein. The lag would be due to the relatively slow reduction of the ferric enzyme by the tetrahydropterin.

Based upon the precedents with TYH and PAH (28–30), it was expected that the initial pterin product with TRH would be the 4-hydroxypterin. The high levels of TRH102–416 have made it possible to demonstrate this directly for the first time, establishing the reaction shown in Scheme 1 for TRH. Moreover, the stoichiometry of one tetrahydropterin consumed per hydroxylation of the physiological substrate BH_4 and the synthetic substrate 6-MePH_4 with both tryptophan and phenylalanine as the amino acid substrate. Thus, based upon the degree of coupling of tetrahydropterin consumption to tyrosine formation, phenylalanine is as good a substrate as tryptophan for TRH102–416. With both TYH and PAH it has commonly been observed that use of nonphysiological substrates results in an excess of tetrahydropterin consumed over amino acid hydroxylated (36, 46, 47). Indeed this is also the case when tyrosine is the substrate for TRH102–416 where there is a 50-fold greater tetrahydropterin oxidation than amino acid hydroxylation.

The steady state kinetic analyses presented here for TRH102–416 provide insight into the substrate specificity of TRH and allow comparison with PAH and TYH. Qualitatively, tyrosine is a poor substrate for TRH102–416, and both tryptophan and phenylalanine are good substrates. Indeed, given that the kinetic parameters in Table III could not be determined at saturating concentrations of the nonvaried substrates due to substrate inhibition, the kinetic parameters for tryptophan and phenylalanine are probably not significantly different. These results can be compared with the substrate specificities of TYH and PAH. It is best to use results obtained with the comparable catalytic domains of the latter enzymes because of the compli-
cations caused by the need for prior activation of wild type PAH. Other than release from cooperative substrate activation of PAH, removal of the regulatory domains of PAH and TYH does not affect the ability of these enzymes to hydroxylate the other aromatic amino acids (17). TYH will hydroxylate phenylalanine (48, 49). Whereas the rate of tetrahydropterin oxidation by TYH in the presence of phenylalanine is comparable to that seen in the presence of tyrosine, only a fraction of the reducing equivalents are used to form tyrosine (17, 49). This is in contrast to the situation with TRH102–416 in which tetrahydropterin oxidation and amino acid hydroxylation are stoichiometric. Tryptophan is also a substrate for TYH, but with a Vmax value only 20% that seen with tyrosine and with a Km value 20-fold higher (50). Thus, tyrosine is clearly the preferred substrate for TYH. PAH is unable to hydroxylate tyrosine, although tyrosine does stimulate a low rate of tetrahydropterin oxidation (17, 49, 51). In contrast, PAH is able to hydroxylate tryptophan (42, 52) but with a Km value a 1000-fold higher and a Vmax value one-tenth that of phenylalanine. Thus, PAH strongly prefers phenylalanine over the other two amino acids as a substrate. While TRH102–416 resembles PAH qualitatively in its ability to hydroxylate only phenylalanine and tryptophan, TRH102–416 shows no discernible preference for its physiological substrate. It is not clear whether there is physiological relevance to this lack of specificity.

In conclusion, the goal of the work presented here was to obtain a form of TRH that would permit study of catalytic properties. The results indicate that rabbit TRH102–416 is a stable, highly active form of TRH that can be expressed to very high levels in E. coli. This mutant enzyme clearly appears to be valid for mechanistic studies of wild type TRH.

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