Influence of Steric Bulk and Electrostatics on the Hydroxylation Regiospecificity of Tryptophan Hydroxylase: Characterization of Methyltryptophans and Azatryptophans as Substrates†

Graham R. Moran,‡,§ Robert S. Phillips,|| and Paul F. Fitzpatrick*,‡,⊥

Department of Biochemistry and Biophysics and Department of Chemistry, Texas A&M University, College Station, Texas 77843-2128, and Department of Chemistry, University of Georgia, Athens, Georgia 30602-2556

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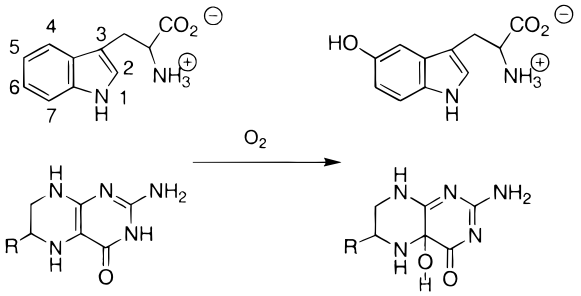
ABSTRACT: Tryptophan hydroxylase is a pterin-dependent amino acid hydroxylase that catalyzes the incorporation of one atom of molecular oxygen into tryptophan to form 5-hydroxytryptophan. The substrate specificity and hydroxylation regiospecificity of tryptophan hydroxylase have been investigated using tryptophan analogues that have methyl substituents or nitrogens incorporated into the indole ring. The products of the reactions show that the regiospecificity of tryptophan hydroxylase is stringent. Hydroxylation does not occur at the 4 or 6 carbon in response to changes in substrate topology or atomic charge. 5-Hydroxymethyltryptophan and 5-hydroxy-4-methyltryptophan are the products from 5-methyltryptophan. These products establish that the hydroxylating intermediate is sufficiently potent to hydroxylate benzylic carbons and that the direction of the NIH shift in tryptophan hydroxylase is from carbon 5 to carbon 4. The effects on the *V*/*K* values for the amino acids indicate that the enzyme is most sensitive to changes at position 5 of the indole ring. The V_{max} values for amino acid hydroxylation differ at most by a factor of 3 from that observed for tryptophan, while the efficiencies of hydroxylation with respect to tetrahydropterin consumption vary 6-fold, consistent with oxygen transfer to the amino acid being partially or fully rate limiting in productive catalysis.

Tryptophan hydroxylase (EC 1.14.16.4) is a member of the family of pterin-dependent hydroxylases. These enzymes are ferrous non-heme monooxygenases that catalyze the hydroxylation of the ring of a specific aromatic amino acid in a reaction that requires tetrahydrobiopterin and molecular oxygen (*1*). The reaction catalyzed by tryptophan hydroxylase (Scheme 1) is the rate-limiting process in the production of serotonin. A number of neurological disorders are associated with altered levels of tryptophan hydroxylase (*2*−*5*). Still, there is a dearth of information regarding the mechanism of this enzyme, due to its low levels in native sources, its low yields from heterologous expression systems, and its marked instability (*6*−*14*). The majority of what is known of the pterin-dependent hydroxylases has come from studies of the other two members of this enzyme family, phenylalanine hydroxylase and tyrosine hydroxylase. The recent expression of a stable truncated form of rabbit tryptophan hydroxylase has provided the first opportunity for biochemical analysis of the reaction mechanism of this important enzyme (*15*). Here we describe the results of an investigation of the substrate specificity and hydroxylation regioselectivity of this recombinant tryptophan hydroxylase using a variety of tryptophan analogues.

Scheme 1



EXPERIMENTAL PROCEDURES

Materials. 6-Methyltetrahydropterin ($6MPH_4$)[1] was from B. Schircks Laboratories. L-Tryptophan, L-5-hydroxytryptophan, DL-4-methyltryptophan, DL-5-methyltryptophan, DL-6-methyltryptophan, DL-7-methyltryptophan, and DL-7-azatryptophan were purchased from Sigma Chemical Co. L-4-Azatryptophan, L-5-azatryptophan, and L-6-azatryptophan were synthesized according to the methods of Sloan and Phillips (*16*) and Phillips et al. (*17*). L-2-Azaisotryptophan

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* To whom correspondence should be addressed. E-mail: fitzpat@tamu.edu. Phone: (409) 845-5487. Fax: (409) 845-9274.

‡ Department of Biochemistry and Biophysics.

§ Present Address: Department of Chemistry, University of Wisconsin, Milwaukee, WI 53201.

|| Department of Chemistry, University of Georgia.

⊥ Department of Chemistry, Texas A&M University.

[1] Abbreviations: $TRH_{102-416}$, rabbit tryptophan hydroxylase lacking amino acids 1−101 and 417−444; $6MPH_4$, 6-methyltetrahydropterin.

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(β-indazoylyl-L-alanine) was prepared by the method of Tanaka et al. (*18*). The concentration of 6-methyltetrahydropterin was determined in 2 M HCl using an ϵ_{266} value of 17 800 M^{-1} cm^{-1}. The concentrations of stocks of tryptophan and 5-hydroxytryptophan were determined in 10 mM HCl using an ϵ_{278} value of 5500 M^{-1} cm^{-1}.

$TRH_{102-416}$ is a truncated monomeric form of rabbit tryptophan hydroxylase lacking 101 residues from the amino terminus and 28 residues from the carboxyl terminus. This enzyme was prepared by the method of Moran et al. (*15*) excluding the ceramic hydroxyapatite step. Enzyme prepared in this manner had a specific activity of 1.6 μmol/mg min at 37 °C and was judged by SDS−polyacrylamide gel electrophoresis to be >90% pure. D-Amino acid oxidase was purified according to the method of Fitzpatrick and Massey (*19*). L-Amino acid oxidase and dihydropterin reductase were purchased from Sigma Chemical Co. Tryptophan 2-monooxygenase was prepared by the method of Emanuele et al. (*20*). Catalase was purchased from Boehringer Mannheim.

Determination of Amino Acid Extinction Coefficients. For DL-5-methyltryptophan, DL-6-methyltryptophan, DL-7-methyltryptophan, and DL-7-azatryptophan, extinction coefficients were determined by using D-amino acid oxidase to measure the concentrations of solutions of known absorbances. D-Amino acid oxidase (~1 nmol) was added to oxidize the D-isomer of racemic mixtures of each amino acid in 100 mM sodium pyrophosphate, pH 8.0, and 0.1 mM FAD. The amount of oxygen consumed was measured using a Yellow Springs Instrument model 5300 biological oxygen monitor thermostated at 25 °C. This was then used as a measure of half the concentration of the racemic mixture. DL-4-Methyltryptophan was not a substrate for D-amino acid oxidase; consequently, L-amino acid oxidase was used in an analogous manner to determine the concentration of this compound. L-2-Azaisotryptophan was not a substrate for L-amino acid oxidase; the concentration of this substrate was determined in an analogous fashion using tryptophan 2-monooxygenase. The extinction coefficients of L-4-azatryptophan, L-5-azatryptophan, L-6-azatryptophan, and L-2-azaisotryptophan were determined by weight in phosphate buffer, pH 8.0.

Determination of Hydroxylation Stoichiometry. The reaction mixture contained 10 μM $TRH_{102-416}$ (prepared in the absence of dithiothreitol), 100 μM amino acid (concentration of the L-isomer), 280 μM oxygen, 200 mM ammonium sulfate, 20 μM ferrous ammonium sulfate, and 50 mM Mes, pH 7.0, at 15 °C in a total volume of 1 mL. Prior to the initiation of turnover, a 100 μL aliquot was withdrawn and mixed with 10 μL of 40% trichloroacetic acid. The reaction was then initiated by the addition of $6MPH_4$ to a final concentration of 50 μM. After the consumption of tetrahydropterin was complete (15−40 s), a second 100 μL aliquot was withdrawn and mixed with acid. These two aliquots were then centrifuged at 12000*g* for 10 min to remove precipitated protein. Supernatants were loaded onto a Waters Nova-Pac reversed-phase C18 HPLC column (150 mm × 3.9 mm ID) using a 20 μL injection loop. For the methyltryptophans and L-2-azaisotryptophan, the mobile phase was 98% 40 mM sodium acetate, pH 3.5, 2% acetonitrile at a flow rate of 1 mL/min. For the azatryptophans, the mobile phase was 10 mM sodium phosphate, pH 7.0, at a flow rate of 1 mL/min. The methyltryptophans and L-2-azaisotryptophan were detected by fluorescence with excitation at their absorbance maxima (see Table 1), monitoring emission at 340 nm. The azatryptophans, which are weakly fluorescent or nonfluorescent, were detected by absorbance at their absorbance maxima (see Table 1). The difference in the area of the substrate peak prior to and after turnover was used to determine the amount of amino acid consumed. The values obtained were independent of the amount of enzyme or $6MPH_4$ used.

Table 1. Spectral Characteristics of Tryptophan Analogues

amino acid	$\lambda_{max(nm)}$	ϵ (mM^{-1} cm^{-1})
4-methyltryptophan[a]	272	8.67
5-methyltryptophan[a]	276	6.12
6-methyltryptophan[a]	280	5.61
7-methyltryptophan[a]	270	6.49
4-azatryptophan[b]	288	14.2
5-azatryptophan[b]	276	15.2
6-azatryptophan[b]	325	9.80
7-azatryptophan[a]	290	6.72
2-isoaztryptophan[a]	290	7.00

[a] 100 mM sodium pyrophospate, pH 8.0. [b] 10 mM sodium phosphate, pH 7.0.

Amino Acid Product Analysis. Reaction mixtures typically contained 3 mg of the L-isomer of the amino acid in 5 mL of 200 mM ammonium sulfate, 100 μM ferrous ammonium sulfate, 0.2 mg/mL catalase, 15 mM β-mercaptoethanol, and 50 mM Mes, pH 7.0. Each mixture was equilibrated at 15 °C and aerated by constant stirring under a stream of air. After the addition of $TRH_{102-416}$ to a final concentration of 50 μM, $6MPH_4$ was added to 150 μM to initiate the reaction. At 2 h intervals, additional quantities of enzyme (125 nmol), $6MPH_4$ (750 nmol), and β-mercaptoethanol (75 μmol) were added. The reaction was allowed to proceed for 2−6 h and was stopped by the addition of 40% trichloroacetic acid to a final concentration of 4%. Denatured protein was removed by centrifugation at 12000*g* for 10 min. Since the major product from 5-methyltryptophan (5-hydroxymethyltryptophan) was found to be unstable in acid, the reaction of this substrate was stopped by filtration through a Millipore Biomax Centrifugal 10 kDa molecular mass cutoff filter. After stopping the reaction, the supernatant or filtrate was then reduced to about 1 mL under reduced pressure and injected onto a Waters μBondapac C18 column (19 × 300 mm). The products were separated at a flow rate of 5 mL/min using a 30 min, 0 to 20% acetonitrile gradient commencing 15 min after injection. For separation of the products of 4-, 6-, 7-methyltryptophan and 2-azaisotryptophan, the mobile phase contained 0.04% trifluoroacetic acid. The mobile phase used for separation of each of the azatryptophan products was 10 mM sodium phosphate, pH 7.0. The products obtained from 5-methyltryptophan were purified using isocratic 6% acetonitrile. All of the products were detected at the near-UV absorbance maximum of the respective amino acid (Table 1) using a Waters model 481 detector. Individual product peaks were collected and lyophilized before being redissolved in 800 μL of deuterium oxide. Proton NMR spectra were recorded using a Varian Unity 500 MHz NMR. A small quantity (0.1−0.2 mg) of each product was then desalted using a analytical Nova-Pac reversed-phase C18 column using a water mobile phase at 1 mL/min. Each was reduced to dryness under reduced pressure and redissolved in dimethyl sulfoxide for electrospray ionization mass spectral analysis using a Vestec 201A

single quadrupole mass spectrometer. The sample was introduced using flow-injection with a mobile phase of acetonitrile and water (1:1).

For substrates that exhibited multiple products, the proportion of each product was measured using a variation of the ortho-phthalaldehyde derivatization method of Hillas and Fitzpatrick (*21*). Prior to the turnover reaction, ammonium sulfate was removed from the enzyme by gel filtration through a Sephadex G-25 column.The conditions of the turnover reaction were 10 μM tryptophan hydroxylase, 500 μM amino acid, and 100 mM Mes, pH 7.0, at 15 °C. The reaction was initiated by the addition of $6MPH_4$ to a final concentration of 300 μM. This method assumes that the fluorescent responses of derivatized tryptophans are essentially equal (*22*).

Enzymatic Assays. Four enzymatic assays were used to determine kinetic parameters. Assay one was the continuous fluorescence assay of Moran and Fitzpatrick (*23*) and was used to determine the kinetic parameters of tryptophan and 7-azatryptophan. The conditions of this assay were 150 μM $6MPH_4$, 278 μM oxygen, 100 μg/mL catalase, 25 μM ferrous ammonium sulfate, 6 mM dithiothreitol, 200 mM ammonium sulfate, and 50 mM Mes, pH 7.0, at 15 °C.

The second assay was the discontinuous HPLC fluorometric assay described by Moran et al. (*15*) and was used for each of the methyltryptophan analogues. In each case, the substrate and product were excited at the substrate near-UV absorbance maximum and their emission detected at 340 nm. The assay conditions were as described above for the continuous fluorescence assay. The product fluorescence signal was standardized by HPLC based on the relative amount of the substrate consumed during turnover. For example, for 4-methyltryptophan a 50 μM sample was partially hydroxylated by tryptophan hydroxylase. Before the initiation of the reaction with $6MPH_4$, a 100 μL aliquot was withdrawn. The progress of the reaction was monitored spectrally at wavelengths where spectral changes due to hydroxylation were maximal (in this case 307 nm). After approximately half the L-isomer of the substrate had been consumed, the reaction was stopped by filtration through a 10 kDa cutoff filter. Aliquots of each sample were then analyzed by HPLC. The relative magnitude of the decrease in the substrate peak was used to determine the decrease in the concentration of the substrate. The concentration of the product was assumed to be equal to the decrease in the concentration of the substrate, allowing the extinction coefficient for the peak due to the product to be calculated.

The third assay coupled the oxidation of $6MPH_4$ during catalysis to the oxidation of NADH using dihydropterin reductase and was based on the method previously described by Fitzpatrick (*24*). Varied concentrations of amino acid were used in assays whose conditions were 100 μM $6MPH_4$, 100 μg/mL catalase, 1 unit/mL dihydropterin reductase, 200 μM NADH, 200 mM ammonium sulfate, 25 μM ferrous ammonium sulfate, and 50 mM Mes, pH 7.0, at 15 °C. Once the autoxidation reaction of the $6MPH_4$ had attained a constant rate, the assay was initiated by the addition of enzyme to a final concentration of 0.5−1 μM. The progress of the reaction was monitored at 340 nm.

Assay four monitored changes in the amino acid spectrum due to hydroxylation. Both 4- and 6-azatryptophan showed sufficient spectral changes when hydroxylated to permit accurate kinetic analyses using increases in absorbance at 328 or 370 nm, respectively. The assay conditions were 150 μM $6MPH_4$, 278 μM oxygen, 200 mM ammonium sulfate, 25 μM ferrous ammonium sulfate, 100 μg/mL catalase, 15 mM β-mercaptoethanol, and 50 mM Mes, pH 7.0 at 15 °C. To determine the extinction coefficients of the 4- and 6-azatryptophan products, a spectrum was recorded of a solution containing all assay ingredients but the substrate. The reaction was then initiated by the addition of the amino acid to a final concentration of 20 μM. Spectra were recorded at 30 s intervals until hydroxylation of the substrate was complete. The spectrum obtained before the addition of substrate was then subtracted from the final spectrum obtained when turnover was complete.

Ab Initio Calculations. Geometry optimizations and Mulliken atomic charges for the indole ring of each tryptophan analogue were calculated using the 6-31G* basis set at the Hartree−Fock level using Spartan software (Wave function Inc.). To reduce the size of the calculations, the 3-methylindole analogue of each amino acid was used for calculations.

RESULTS

Product Analyses. To understand how the structure of the amino acid substrate influences hydroxylation regioselectivity in tryptophan hydroxylase, a number of tryptophan analogues have been characterized as substrates for tryptophan hydroxylase. Methyltryptophans were selected as substrates to probe steric interactions, since the methyl group should not alter the reactivity of the nonsubstituted indole ring carbons greatly. Azatryptophans were selected because they are isosteric with tryptophan yet the indole ring carbons should have dramatically altered reactivities due to the pyridyl nitrogen.

The amino acid products which result when each of these analogues is used as a substrate for tryptophan hydroxylase were determined by carrying out large-scale reactions and isolating the products. For each amino acid, approximately 5−10 μmol of the amino acid product(s) was purified by HPLC, and its structure determined by NMR and mass spectrometry. In most cases, only one amino acid product was observed. Electrospray mass spectrometry of the starting materials and products demonstrated that each product had acquired 16 mass units relative to the starting material, consistent with the addition of a single oxygen atom (results not shown). Figures 1, 2, and 4 show the products of hydroxylation of each of these substrates and Table 2 lists the NMR chemical shifts of the protons of each product.

Each of the methyltryptophans was hydroxylated by tryptophan hydroxylase. With 4-, 6-, and 7-methyltryptophan, the 5-hydroxy amino acid was the exclusive product (Figure 1). In contrast, two products were observed with 5-methyltryptophan as a substrate for tryptophan hydroxylase. The major product (>99%) was 5-hydroxymethyltryptophan. The second product was produced at levels too low for characterization by NMR, but could be identified as 5-hydroxy, 4-methyltryptophan by HPLC, using the 5-hydroxy, 4-methyltryptophan produced from 4-methyltryptophan as a standard.

The amino acid products when azatryptophans are substrates for tryptophan hydroxylase are shown in Figure 2. Hydroxylated amino acids were produced from 4-, 6-, and

Table 2: Proton Nuclear Magnetic Resonances of the Products of Hydroxylation of Tryptophan Analogues by Tryptophan Hydroxylase[a]

	proton (multiplicity, shift (ppm), coupling (Hz))					
amino acid	H1	H2	H4	H5	H6	H7
5-hydroxy,4-methyltryptophan		s, 7.24			d, 7.26, 8.5	d, 6.89, 8.5
5-hydroxy-methyltryptophan		bs, 7.33	s, 7.71		d, 7.53, 10.0	dd, 7.28, 10.0 + 1.4
5-hydroxy,6-methyltryptophan		s, 7.18	s, 7.11			q, 7.29, 0.5
5-hydroxy,7-methyltryptophan		s, 7.23	d, 6.71, 1.0		d, 6.98, 1.0	
5-hydroxy,4-azatryptophan		s, 7.32			d, 6.384, 9.0	d, 7.89, 9.0
5-hydroxy,6-azatryptophan		s, 7.63	s, 7.96			bs, 6.70
5-hydroxy,7-azatryptophan		s, 7.35	d, 7.60, 2.5		d, 7.92, 2.5	
6-hydroxy-2-azaisotryptophan	d, 8.08, 0.9		dd, 7.73, 8.8 + 0.6	dd, 6.88, 1.9 + 8.7		dd, 6.99, 1.1 + 1.9
5-hydroxy-2-azaisotryptophan	d, 8.05, 0.9		dd, 7.21, 2.5 + 0.5		dd, 7.17, 2.0 + 9.0	ddd, 7.55, 1.0 + 1.0 + 9.0

[a] All NMR spectra were recorded in D_2O at 25 °C: bs, broad singlet; s, singlet; d, doublet; q, quartet.

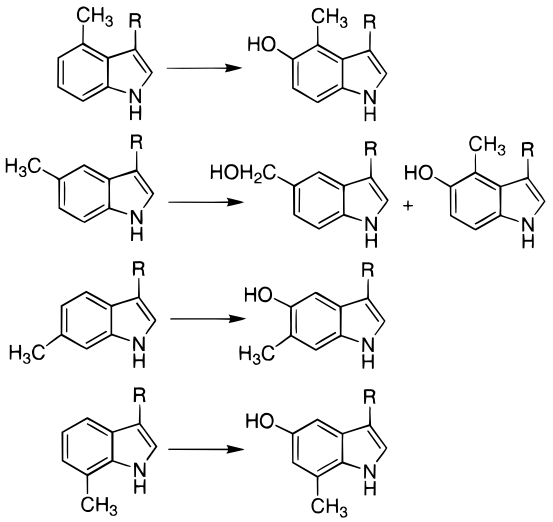


FIGURE 1: The products of hydroxylation of methyltryptophans by tryptophan hydroxylase. In each structure R═CH_2CHNH_3COO.

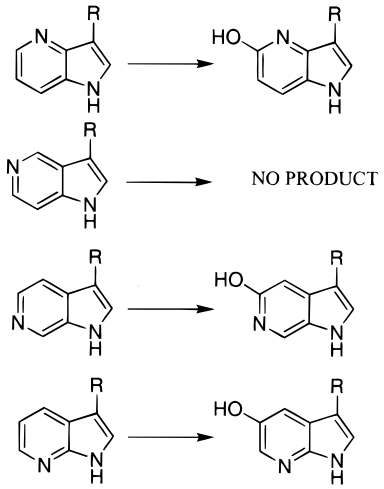


FIGURE 2: The products of hydroxylation of azatryptophans by tryptophan hydroxylase. In each structure R═CH_2CHNH_3COO.

7-azatryptophan, but no product from 5-azatryptophan could be detected. As was the case with the methyltryptophans, hydroxylation occurred exclusively at the 5-carbons for the azatryptophans which were substrates. In contrast, two products in approximately equal proportion were observed when 2-azaisotryptophan was used as a substrate (Figure 3 and Table 2). These were identified as 5-hydroxy-2-azaisotryptophan and 6-hydroxy-2-azaisotryptophan. This was the only substrate for which the site of hydroxylation changed.

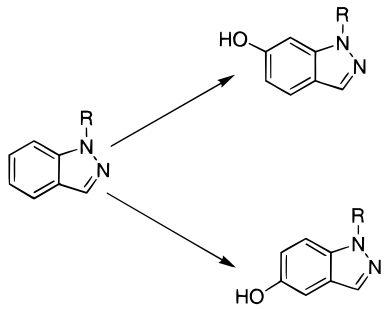


FIGURE 3: The products of hydroxylation of 2-azaisotryptophan by tryptophan hydroxylase. R═CH_2CHNH_3COO.

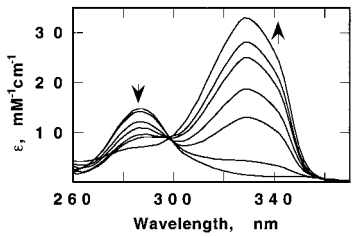


FIGURE 4: Spectral changes upon hydroxylation of 4-azatryptophan by tryptophan hydroxylase. Traces were recorded at 0, 2, 32, 62, 122, 182, and 2000 s. The reaction was carried out using 5 μM enzyme in 100 μM 6-methyltetrahydropterin, 320 μM oxygen, 200 mM ammonium sulfate, 15 mM β-mercaptoethanol, 20 μM ferrous ammonium sulfate, 50 mM MES, pH 7.0, at 15 °C. Prior to the initiation of turnover, a baseline spectrum was recorded with all reaction substituents except 4-azatryptophan. The reaction was then commenced by the addition of 4-azatryptophan to 18.5 μM.

The hydroxylated products from 4- and 6-azatryptophan absorb strongly at 328 nm (31.3 mM^{-1} cm^{-1}) or 370 nm (9.8 mM^{-1} cm^{-1}), respectively (Figure 4). On the basis of the strong absorbance of the product, 4-azatryptophan has potential for the development of a simple and exceedingly sensitive continuous assay for tryptophan hydroxylase. The large extinction coefficient of 5-hydroxy,4-azatryptophan would give reliable detection down to 50 nM on most spectrophotometers.

Kinetic Parameters. Steady-state kinetic parameters for each of the tryptophan analogues as substrates for tryptophan hydroxylase are listed in Table 3. The rates of formation of the hydroxylated amino acids were determined using several assays, with the assay used depending on the properties of the individual amino acid. A continuous fluorescence assay was used for tryptophan and 7-azatryptophan. A discontinuous fluorescence HPLC assay was used for methyltryptophans, since their product spectra are not sufficiently different from the substrate spectra to permit the use of the

Table 3: Apparent Kinetic Parameters for Tryptophan Analogues as Substrates for Tryptophan Hydroxylase[a]

amino acid	K_m (μM)	$V_{amino\ acid}$[b] (min^{-1})	$V/K_{amino\ acid}$[b] ($mM^{-1}\ min^{-1}$)	V_{Pterin}[c] (min^{-1})	amino acid hydroxylated/ tetrahydropterin consumed[d]
tryptophan[e]	2.1 ± 0.1	12.4 ± 0.1	5800 ± 200	12.4 ± 0.1	1.10 ± 0.06
4-methyltryptophan[f]	994 ± 192	16.4 ± 2.1	16.4 ± 1.1	53.8 ± 6.9	0.30 ± 0.007
5-methyltryptophan[f]	>16000	nd[g]	0.479 ± 0.005	nd	0.15 ± 0.015
6-methyltryptophan[f]	15.1 ± 0.4	4.4 ± 0.1	210 ± 20	9.2 ± 0.1	0.48 ± 0.03
7-methyltryptophan[f]	37.5 ± 0.3	18.9 ± 1.0	500 ± 70	24.9 ± 1.3	0.758 ± 0.02
4-azatryptophan[h]	262 ± 13	21.5 ± 0.7	82 ± 2	57.1 ± 1.7	0.38 ± 0.03
6-azatryptophan[h]	295 ± 12	32 ± 0.7	93 ± 2	77.6 ± 1.7	0.35 ± 0.01
7-azatryptophan[e]	66.2 ± 7.1	15.1 ± 0.9	230 ± 10	15.1 ± 0.9	1.15 ± 0.14
2-isoazatryptophan[i]	86.5 ± 33	13.0 ± 2.0[j]	150 ± 30[j]	19.8 ± 3.0	0.66 ± 0.069

[a] Conditions: 200 mM ammonium sulfate, 25 μM ferrous ammonium sulfate, 50 mM MES, pH 7.0, 15 °C. [b] On the basis of rates of amino acid hydroxylation at 100−150 μM $6MPH_4$. [c] The rate of pterin consumption at the maximal rate of amino acid hydroxylation calculated from the rates of amino acid hydroxylation and the relative stoichiometries of amino acid hydroxylation and tetrahydrotperin oxidation. [d] Determined using limiting tetrahydropterin. [e] Measured using the continuous assay of ref *23*. [f] Measured using the discontinuous HPLC assay described in Experimental Procedures. [g] Not determined due to high K_m value. [h] Measured using the extinction coefficient of the product. [i] Measured using the dihydropterin reductase coupled assay described in Experimental Procedures. [j] Calculated from rates of tetrahydropterin oxidation and the relative stoichiometries of amino acid hydroxylation and tetrahydrotperin oxidation.

first assay. A coupled assay which measured the rate of tetrahydropterin oxidation was used to determine kinetic parameters for 2-azaisotryptophan. The kinetic parameters of both 4-aza- and 6-azatryptophan were determined by monitoring the absorbances due to the products, taking advantage of the large spectral changes illustrated in Figure 4.

The *V*/*K* values for hydroxylation of 4- and 5-methyltryptophan are 3 and 4 orders of magnitude smaller, respectively, than that for tryptophan. In the case of 5-methyltryptophan, the rate of turnover varied directly with the concentration of the amino acid up to 1.6 mM, the limit of solubility, so that only a lower limit on the K_m value can be estimated. In contrast, the *V*/*K* values for 6- and 7-methyltryptophan are only 1 order of magnitude smaller than that for tryptophan. A similar trend is observed with the azatryptophans. As noted above, 5-azatryptophan is not hydroxylated by tryptophan hydroxylase. In addition, no enzyme-catalyzed tetrahydropterin oxidation could be detected in the presence of concentrations of this substrate up to 1.2 mM. For the other azatryptophans, the *V*/*K* values increase as the pyridyl nitrogen resides further from the 5-position. The *V*/*K* value for 2-azaisotryptophan is 40-fold less than that of tryptophan. The V_{max} values for hydroxylation of the all tryptophan analogues investigated differ at most by a factor of 3 from that observed with tryptophan, with the azatryptophans showing larger V_{max} values than tryptophan.

When nonphysiological amino acids are used as substrates for the pterin-dependent hydroxylases, the amount of tetrahydropterin oxidized can exceed the amount of amino acid hydroxylated due to unproductive turnover (*1*). For each of the tryptophan analogues described here, the relative stoichiometries of tetrahydropterin oxidized and amino acid hydroxylated were determined. This was done by utilizing limiting amounts of $6MPH_4$ in the presence of saturating concentrations of the amino acid. High levels of enzyme were used to ensure that the tetrahydropterin was consumed rapidly in order to minimize autoxidation. The results of these analyses are given in Table 3. With all of the compounds except 7-azatryptophan, an excess of tetrahydropterin is consumed unproductively. With the methyltryptophans, the amount of unproductive turnover is maximal for 5-methyltryptophan and decreases as the methyl group is located farther from the site of hydroxylation. Similar results are seen with the azatryptophans, in that hydroxylation is relatively inefficient with 4- and 6-azatryptophan and normal with 7-azatryptophan. The V_{max} values for tetrahydropterin consumption can be calculated from the V_{max} values for amino acid hydroxylation and the relative stoichiometries of amino acid hydroxylation and tetrahydropterin oxidation; these values are also given in Table 3. With 4-methyltryptophan and 4-and 6-azatryptophan, the total rate of tetrahydropterin oxidation is significantly greater than the rate of turnover with tryptophan itself.

Table 4: Mulliken Atomic Charges for Tryptophan Analogues[a]

amino acid	C4	C5	C6	C7	CH_3
tryptophan	−0.20	−0.23*[b]	−0.21	−0.22	
4-methyltryptophan	0.06	−0.26*	−0.20	−0.23	−0.50
5-methyltryptophan	−0.24	0.02*	−0.23	−0.21	−0.50*
6-methyltryptophan	−0.19	−0.25*	0.04	−0.26	−0.51
7-methyltryptophan	−0.21	−0.21*	−0.25	0.05	−0.59
4-azatryptophan	−0.58	−0.04*	−0.28	−0.16	
5-azatryptophan	0.08	−0.55	−0.06	−0.28	
6-azatryptophan	−0.26	−0.04*	−0.54	0.06	
7-azatryptophan	0.14	−0.29*	0.06	−0.60	
2-isoazatryptophan	−0.21	−0.23*	−0.23*	−0.18	

	C2	C3	C4	C5
phenylalanine	−0.23	−0.19	−0.21*	−0.19
tyrosine[c]	−0.20	−0.26*	0.40	−0.26*

[a] Charge densities were calculated using the 6-31G* basis set at the Hartree−Fock level. [b] The asterisks indicate the sites of hydroxylation. [c] Hydroxylation site is for hydroxylation by tyrosine hydroxylase. The densities at C3 and C5 are average values. The specific values at the two positions differ depending on the orientation of the hydroxyl proton.

Atomic Charges. The atomic charges of the indole rings of the individual tryptophan analogues were calculated from the optimized structures of their respective 3-methylindole derivatives. Preliminary calculations using geometrically optimized tryptophan and 3-methylindole showed that the distribution of charge is unaffected by the removal of the amino acid portion of the molecule. Table 4 lists the calculated atomic charges for each of the benzene or pyridine ring atoms of the substrates. The methyl groups of the methyltryptophans do not greatly influence the atomic charges of the nonsubstituted indole ring atoms. In each case, however, the carbon to which the methyl group is bonded is electron deficient. The azatryptophans are isosteric with tryptophan yet have very different patterns of atomic charge.

The presence of the electronegative nitrogen in the ring results in greatly decreased electron density at the adjacent carbons. 7-Azatryptophan has the highest electron density at position 5. 2-Azaisotryptophan has approximately uniform charge distribution for carbons 4, 5, 6 and 7. The atomic charges for phenylalanine and tryptophan are also listed for comparison.

DISCUSSION

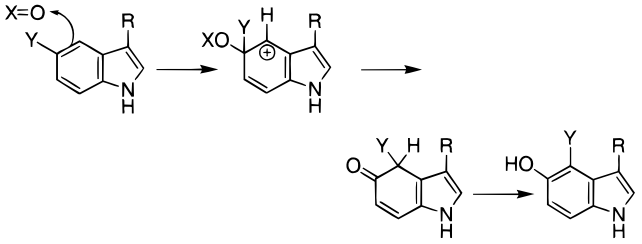
Two classes of tryptophan analogues have been characterized as substrates for tryptophan hydroxylase to examine the basis for the hydroxylation regiospecificity of the enzyme. Analogues with methyl substituents were used as probes for the sensitivity of the reaction to steric bulk, while analogues with additional ring nitrogens were used as probes of the reaction to changes in atomic charge. With the exception of 5-azatryptophan, all of the compounds were substrates for tryptophan hydroxylase. To a large extent, the site of hydroxylation was unaffected by either modification. All of the methyltryptophans were hydroxylated at position 5 of the indole ring, demonstrating that the steric bulk of the methyl group did not disrupt the binding of the amino acid sufficiently to alter the regiospecificity. Similarly, for the three azatryptophans which were substrates, the site of hydroxylation was unchanged despite large changes in the electron density of carbon 5 due to the presence of the additional nitrogen. The results with both series of analogues suggest that the amino acid has a single binding mode in the active site.

The only amino acid for which the site of hydroxylation was altered was 2-isoazatryptophan. This is also the only amino acid which did not have a nitrogen atom at position 1 of the ring, raising the possibility that this nitrogen is critical for proper positioning of the indole ring. This nitrogen is the only atom in the ring which would be expected to form a hydrogen bond with an amino acid residue in the protein, so that it could certainly play a dominant role in properly positioning the amino acid for hydroxylation.

The only amino acid examined here which was not a substrate for tryptophan hydroxylase was 5-azatryptophan. In addition, there was no unproductive oxidation of $6MPH_4$ in the presence of this amino acid, suggesting that binding of this amino acid to tryptophan hydroxylase is quite weak. The analogous pyridylalanines bind weakly to bovine tyrosine hydroxylase (*24*), suggesting that the substitution of a nitrogen at the site of hydroxylation generally weakens binding to this family of enzymes.

Two different hydroxylated amino acids are produced when 5-methyltryptophan is a substrate for tryptophan hydroxylase. The major product is 5-hydroxymethyltryptophan, indicating that the hydroxylating intermediate of tryptophan hydroxylase is sufficiently potent to hydroxylate benzylic carbons. This is similar to what is observed in the hydroxylation of 4-methylphenylalanine to 4-hydroxymethylphenylalanine by phenylalanine hydroxylase (*25*) and tyrosine hydroxylase (*21*) and supports a model in which the oxygenating intermediate is the same in all three enzymes. The minor product from 5-methyltryptophan is 4-methyl-5-hydroxytryptophan, in which the 5-methyl has migrated to the adjacent 4-carbon. The formation of this compound requires an NIH shift of a methyl group. It also shows that the direction of shift is from carbon 5 to carbon 4, as previously concluded by Renson et al. from studies of [5-^{3}H]-tryptophan (*26*). The occurrence of NIH shifts involving methyl, chloride, and bromide moieties by tyrosine hydroxylase was previously taken as support for an electrophilic aromatic substitution mechanism for hydroxylation, in which the initial attack of the oxygenating intermediate forms a cation (*21*). The NIH shift of a methyl group with tryptophan hydroxylase is consistent with a similar mechanism for that enzyme. Scheme 2 is a mechanism for tryptophan hydroxylase based on the present results and on earlier studies of tyrosine hydroxylase (*21*).

Scheme 2



The *V*/*K* values provide a more sensitive measure of the effects of substrate structure on reactivity. The methyltryptophans probe the steric interaction between the enzyme and the indole ring of the amino acid substrate. As noted above, no turnover can be detected with 5-azatryptophan as substrate, while the methyl group in 5-methyltryptophan decreases the *V*/*K* value by 4 orders of magnitude. The *V*/*K* value for 4-methyltryptophan is decreased almost 3 orders of magnitude, while those for 6-methyl-and 7-methyltryptophan are much closer to that of the native substrate. In addition, hydroxylation is least efficient when the methyl group resides on the 4- or 5-carbon of the indole ring. These results suggest that carbons 4 and 5 of the indole ring are tightly packed in the active site, in close proximity to the hydroxylating intermediate. Substituents in these positions would prevent correct binding of the indole ring and disrupt the hydroxylation reaction. The effects become less pronounced as the methyl group is moved to the 6- and 7-carbons, suggesting that there is more flexibility in this region of the active site.

The *V*/*K* values for the azatryptophans show a similar trend. Catalysis is most disrupted when the pyridyl nitrogen is located at positions 4 or 6, with no detectable reaction occurring with 5-azatryptophan. The *V*/*K* values for 4-aza and 6-azatryptophan are 50- and 60-fold smaller, respectively, than that observed for tryptophan. This is a much smaller effect than was seen with the methyltryptophans, suggesting that the effects with the latter substrates are due to steric interactions. The weaker interactions with the azatryptophans may be due to a decreased interaction between the more polar azaindole ring and the hydrophobic active site. The structures of the homologous catalytic domains of tyrosine and phenylalanine hydroxylase show that these active sites are primarily hydrophobic (*27*, *28*). The few charged amino acid residues in the active sites of all three enzymes have been implicated as iron ligands or in binding polar or charged moieties on the amino acid and tetrahydropterin substrates (*27*, *29*, *30*).

The V_{max} values for hydroxylation of all the individual amino acids are within a factor of 3 of that observed for

tryptophan. However, in some cases, the rate of tetrahydropterin oxidation exceeds that seen with tryptophan by severalfold due to the large fraction of unproductive turnover. Previous studies of the homologous pterin-dependent enzyme tyrosine hydroxylase strongly support a kinetic mechanism in which there is a rate-limiting formation of the hydroxylating intermediate followed by relatively rapid hydroxylation of the amino acid substrate (*21*, *24*). Changing the reactivity of the amino acid substrate has little effect on the overall rate of tetrahydropterin consumption by tyrosine hydroxylase, although it does alter the relative amount of amino acid hydroxylated. Catalysis by tryptophan hydroxylase would be expected to show the same two individual reactions in catalysis. In contrast to the situation with tyrosine hydroxylase, the rate of tetrahydropterin consumption is significantly greater than that for tryptophan with several of the amino acids in Table 3. This is consistent with the rate-limiting step in productive catalysis being a step in the hydroxylation of the substrate rather than formation of the hydroxylating intermediate. In the case of 4- and 6-azatryptophan and 4-methyltryptophan, the disrupted orientation of the substrate in the active site destabilizes the hydroxylating intermediate so that it can break down unproductively. If the unproductive breakdown is more rapid than productive hydroxylation of the amino acid, the rate of turnover can increase. Kinetic isotope effects with deuterated tryptophans also support a model in which oxygen addition to the amino acid is slower than formation of the hydroxylating intermediate.[2] Thus, for highly uncoupled substrates, the rate of pterin consumption is an approximate indication of the rate of the reaction of the enzyme with molecular oxygen. This rate appears to have a limit that is similar to the rates of turnover for tyrosine hydroxylase and phenylalanine hydroxylase, both of which are thought to have the reaction with molecular oxygen as the rate-limiting step in catalysis. In addition, the maximal rate of turnover observed with phenylalanine as a substrate for tryptophan hydroxylase is 100 min^{-1}, similar to the fastest rates of tetrahydropterin consumption observed here (*15*).

In summary, these studies have revealed that tryptophan hydroxylase is most affected by substituents at positions 4 and 5 of the indole ring. The hydroxylating intermediate of tryptophan hydroxylase has stringent hydroxylation regioselectivity and will not hydroxylate at positions 4 or 6 in response to changes in substrate atomic charge distribution or steric obstruction at carbon 5. The hydroxylating intermediate of tryptophan hydroxylase is capable of oxygenating deactivated aromatic and benzylic carbons.

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