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On the Catalytic Mechanism of Tryptophan Hydroxylase

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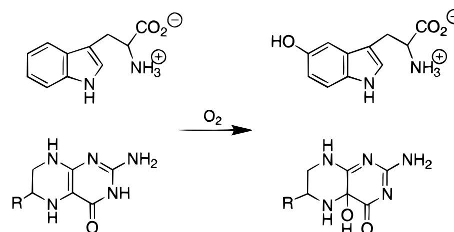
Abstract: Tryptophan hydroxylase catalyzes the hydroxylation of tryptophan using tetrahydrobiopterin and molecular oxygen. With tyrosine as a substrate, the amount of C4a-hydroxypterin formed greatly exceeds the amount of dihydroxyphenylalanine formed, consistent with oxygen−oxygen bond cleavage occurring in a step prior to amino acid hydroxylation. With L-indole-$^2H_5$-tryptophan, L-4-^{2}H- or L-5-^{2}H-tryptophan as substrate there is no isotope effect on the V/K value for tryptophan. There is an inverse isotope effect on the V_{max} value with L-indole-$^2H_5$-tryptophan and L-5-^{2}H-tryptophan, but no effect with L-4-^{2}H-tryptophan. Comparison of the measured isotope effects with values of calculated secondary equilibrium isotope effects for tryptophan hydroxylation indicate that the results are most consistent with the formation of a cationic species. Retention of the isotopic label from L-5-^{2}H-tryptophan in the product confirms that an NIH shift occurs in tryptophan hydroxylase and shows that the direction of shift is from carbon 5 to carbon 4. The degree of retention of the deuterium is higher when the deuterium is initially on carbon 4 rather than carbon 5.

Tryptophan hydroxylase (TRH, EC 1.14.16.4) is a member of a family of closely related pterin-dependent aromatic amino acid hydroxylases which includes phenylalanine hydroxylase and tyrosine hydroxylase.[1] Each of these enzymes catalyzes the hydroxylation of a specific aromatic amino acid, requires ferrous iron for activity, and has tetrahydrobiopterin and molecular oxygen as the other substrates. The physiological function of TRH is to hydroxylate tryptophan to form 5-hydroxytryptophan (Scheme 1). In mammalian metabolism this is the rate-limiting process in the production of the neurotransmitter serotonin.

There are several outstanding mechanistic questions regarding the catalytic mechanism of tryptophan hydroxylase. These include the identity of the hydroxylating intermediate, the mechanism of oxygen addition to the aromatic ring of the amino acid, and the identity of the rate-limiting step in catalysis. Several possibilities have been suggested for the hydroxylating intermediate in this family of enzymes, including a pterin peroxide, a high valence iron oxo species, and peroxypterin iron intermediate; a critical distinction among these proposals is the relative timing of the cleavage of the oxygen−oxygen bond in molecular oxygen or a peroxide and of the formation of the new bond between oxygen and the amino acid substrate. A common characteristic of the pterin-dependent hydroxylases

Scheme 1



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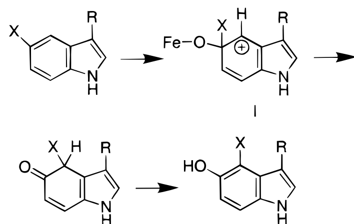
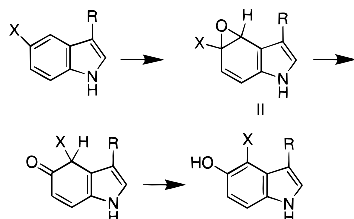
Scheme 2



Scheme 3



germane to the mechanism of oxygen addition is that an NIH shift occurs upon hydroxylation of an amino acid.[2-4] Thus, 5-^{3}H-tryptophan is converted to 4-^{3}H-5-hydroxytryptophan by TRH.[4] Two principle mechanisms have been proposed to explain the NIH shift. Guroff et al.[5] initially proposed an electrophilic aromatic hydroxylation mechanism with an arenium cation (**I**) as the first intermediate (Scheme 2); more recent studies of tyrosine hydroxylase are consistent with formation of such a cationic intermediate upon oxygen addition.[6] Alternatively, oxygen addition could initially form an arene oxide (**II**), as shown in Scheme 3; the ability of phenylalanine hydroxylase to catalyze epoxide formation is consistent with such a mechanism,[7] although results with tyrosine hydroxylase do not support the involvement of an arene oxide with that enzyme.[8] Studies with isotopically labeled and alternative substrates suggest that the reaction of the hydroxylating intermediate with the amino acid is not rate-limiting for either phenylalanine or tyrosine hydroxylase;[9,10] in contrast, recent studies utilizing tryptophan analogues as substrates for TRH suggest that this step may be rate-limiting for that enzyme.[11]

Mechanistic studies of TRH have been greatly hindered by the marked instability and low specific activity of the enzyme from a variety of sources.[12-18] Recently, a form of TRH having just the amino acids of the catalytic domain has provided an enzyme amenable to mechanistic investigation.[19] The present contribution describes the use of this enzyme to address these mechanistic questions.

Experimental Procedures

Materials. 6-Methyltetrahydropterin (6MPH$_4$) and tetrahydrobiopterin were from B. Schircks Laboratories. Tryptophan and 5-hydroxytryptophan were purchased from Sigma Chemical Co. ^{2}H$_5$-Indole-tryptophan was purchased from Cambridge Isotope Laboratories, Inc. The syntheses of 4-^{2}H- and 5-^{2}H-indole were by the method of Bosin et al.[20] They were purified using a silica gel 60 column (2.5 × 15 cm) with a toluene:hexane (1:1) mobile phase. The method of Phillips et al.[21] was used to synthesize L-4-^{2}H- and L-5-^{2}H-tryptophan from the respective deuterated indoles. The amino acids were purified using a 4.9 × 300 mm Prep Novapac HPLC column with water as the mobile phase. Quinonoid dihydrobiopterin was formed immediately before use by oxidation of tetrahydrobiopterin with bromine. The catalytic core of rabbit tryptophan hydroxylase, a mutant protein lacking 101 residues from the amino terminus and 28 residues from the carboxyl terminus of the wild-type protein, was prepared by the method of Moran et al.,[19] excluding the ceramic hydroxyapatite step. Enzyme prepared in this manner has a specific activity of 1.6 μmole/mg-min at 37 °C and was judged to be greater than 90% pure by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The catalytic domain of human phenylalanine hydroxylase was generously provided by Dr. S. Colette Daubner. The concentration of 6MPH$_4$ was determined in 2 M HCl with an ϵ_{266} value of 17 800 M^{-1} cm^{-1}. The concentration of stocks of tryptophan and 5-hydroxytryptophan were determined in 10 mM HCl with an ϵ_{278} value of 5500 M^{-1} cm^{-1}.

Analysis of Deuterium Retention in 5-Hydroxytryptophan. The reaction mixture contained 0.2 μmole TRH and 5 mg of L-5-^{2}H-tryptophan or L-4-^{2}H-tryptophan in 10 mL of 400 mM ammonium sulfate, 15 mM β-mercaptoethanol, 100 μM ferrous ammonium sulfate, 600 μM 6MPH$_4$, 100 mM MES, pH 7.0. The sample was incubated for 2 h at 15 °C with constant stirring under a stream of air. The reaction was stopped by filtering the sample through a Millipore Biomax 10 kDa filter. The eluate was then lyophilized to dryness and redissolved in 1.5 mL of water. The 5-hydroxytryptophan was purified by HPLC, using a 19 × 300 mm μBondapac C18 column with a gradient from 0 to 30% acetonitrile in water and detection by absorbance at 278 nm. The products were collected, lyophilized, and redissolved in 700 μL of deuterium oxide. The deuterium content and position were determined using a Varian Unity 500 MHz NMR.

Kinetic Isotope Effects. Initial rates of hydroxylation of tryptophan to hydroxytryptophan were determined by using the fluorescence assay of Moran and Fitzpatrick.[22] An Applied Photophysics stopped-flow spectrofluorometer was used to mix 50 nM enzyme in air-saturated 400 mM ammonium sulfate, 7 mM dithiothreitol, 25 μg/mL catalase, 50 μM ferrous ammonium sulfate, 100 mM MES, pH 7.0, with solutions of air-saturated tryptophan containing 300 μM 6MPH$_4$ in 10 mM HCl. Isotope effects were also measured at high oxygen concentration by equilibrating the substrate solution with oxygen gas prior to mixing, giving a final oxygen concentration of 830 μM. To calculate the isotope effects, initial rate data were fit to eqs 1−3 using the program *NonLin for Macintosh* (Robelko Software, Carbondale, IL), a Macintosh compatible version of software developed by Johnson and Frasier.[23] The confidence limits of the optimized parameters were set at 67%. Equation 1 describes data for which there are isotope effects on both

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the V_{max} and the V/K_{trp} values, eq 2 describes data for which there is an isotope effect on the V_{max} value only, and eq 3 describes data for which there is an isotope effect on the V/K_{trp} value only. Here v is the initial rate, V is the limiting velocity of the reaction, K_a is the K_m value for tryptophan, F_i is the mole fraction of deuterium in tryptophan, A is the concentration of tryptophan, and E_v and E_{vk} are the isotope effects on the V_{max} and V/K_{trp} values, respectively.

$$v = \frac{K_a(1 + F_i(E_{vk} - 1)) + A(1 + F_i(E_v - 1))}{VA} \quad (1)$$

$$v = \frac{K_a + A(1 + F_i(E_v - 1))}{VA} \quad (2)$$

$$v = \frac{K_a(1 + F_i(E_{vk} - 1)) + A}{VA} \quad (3)$$

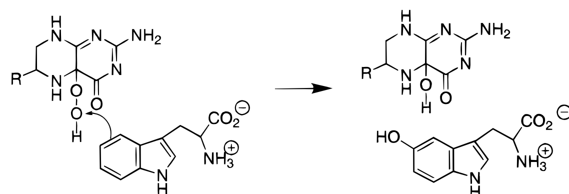
Calculation of Equilibrium Isotope Effects. A Silicon Graphics Power Challenge 10000 XL was used for all calculations. To reduce the time required for calculations, the 3-methylindole analogue of each structure was optimized. Structures of the proposed reaction coordinates were refined in Gaussian 94[24] using the 6-31G** basis set at the Hartree−Fock level. The vibrational frequencies of all reactants and products were then calculated at the same level of theory. Energy minima were verified by the lack of negative frequencies. Equilibrium isotope effects were calculated using the Bigeleisen equation[25] implemented via QUIVER[26] from the vibrational frequencies of the isotopically labeled and unlabeled compounds.

Burst Kinetics. TRH (20 μM) in 400 mM ammonium sulfate, 2 mM dithiothreitol, 100 μg/mL catalase, 50 μM ferrous ammonium sulfate, 100 mM MES, pH 7.0, was mixed in the stopped-flow spectrofluorometer with 120 μM tryptophan, 120 μM 6MPH$_4$, 10 mM HCl, 12 mM dithiothreitol at 15 °C. The final concentration of oxygen was 295 μM. The increase in fluorescence emission above 305 nm upon excitation at 300 nm was used to monitor formation of hydroxytryptophan.

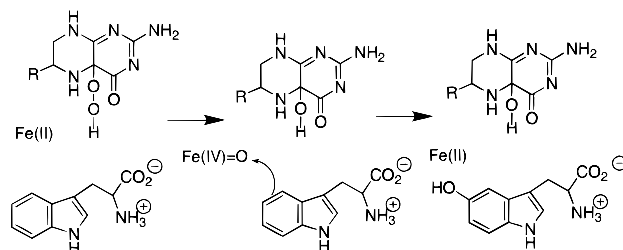
Analysis of Pterin Products with Tyrosine as Substrate. The reaction mixture contained 10 μM TRH (prepared in the absence of dithiothreitol), 650 μM tyrosine, 278 μM oxygen, 200 mM ammonium sulfate, 50 μM ferrous ammonium sulfate, 50 mM MES, pH 7.0, at 15 °C. The reaction was initiated by the addition of tetrahydrobiopterin to a final concentration of 120 μM. Absorbance spectra between 219 and 447 nm were recorded at 1 s intervals with a Hewlett-Packard 8453 diode array spectrophotometer. The spectral changes were simulated using the program SPECFIT (Spectrum Software Associates) and the mechanism of Scheme 6. The spectra of tetrahydrobiopterin and quinonoid dihydrobiopterin used in the simulations were determined directly from synthetic samples of these compounds. To obtain the spectrum of 4a-hydroxybiopterin, the catalytic domain of human phenylalanine hydroxylase[27] (10 μM) was added to 100 μM tetrahydrobiopterin and 3 mM phenylalanine in 20 mM Tris acetate, pH 8.0, 25 °C. The values of k_1-k_5 were adjusted until the simulated absorbance changes agreed with the experimental values.

To determine the total amount of dihydroxyphenylalanine formed, 100 μL of the reaction mixture was withdrawn and quenched into 10 μL of 40% trichloroacetic acid after 30 s. After the sample was centrifuged to remove precipitated protein, the amount of dihydroxyphenylalanine was quantitated by HPLC using a 3.9 × 150 mm Novapac C18 column with 40 mM sodium acetate, pH 3.5, as the mobile phase at a flow rate of 1 mL/min. The dihydroxyphenylalanine was detected using a Waters 470 fluorescence detector with excitation at 280 nm and detection at 310 nm.

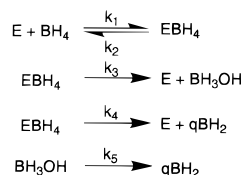
Scheme 4



Scheme 5



Scheme 6



Results

Characterization of Pterin Products with Tyrosine as Substrate. With either tryptophan or phenylalanine as substrate for TRH, the tetrahydropterin is quantitatively converted to a hydroxypterin concomitantly with hydroxylation of tryptophan, as shown in Scheme 1. In contrast, when tyrosine is used as substrate, the amount of dihydroxyphenylalanine formed is only 1.2% the amount of tetrahydropterin consumed.[19] The various proposals for the hydroxylating intermediate make different predictions as to the amount of hydroxypterin which could be formed with this substrate. If a peroxypterin is the hydroxylating intermediate, cleavage of the oxygen−oxygen bond is concerted with hydroxylation of the amino acid, so that the amount of hydroxypterin formed should equal the amount of dihydroxyphenylalanine (Scheme 4). In contrast, if the oxygen−oxygen bond must be cleaved in order to form the actual hydroxylating intermediate (Scheme 5), unproductive breakdown of the latter could result in an excess of hydroxypterin over dihydroxyphenylalanine. Formation of the hydroxypterin can readily be detected at its absorbance maximum of 246 nm.[28] Accordingly, the near UV spectral changes which occur when tetrahydrobiopterin and tyrosine are substrates for TRH were determined. To ensure that the rate of formation of the hydroxypterin was significantly greater than its rather facile dehydration, high concentrations of TRH (10 μM) were utilized with a limiting amount of tetrahydrobiopterin (120 μM). The spectral changes which occurred during the first 70 s of the reaction are shown in Figure 1A. There is a clear increase in absorbance at 246 nm which reaches a maximum after 25 s, consistent with transient formation of hydroxybiopterin.

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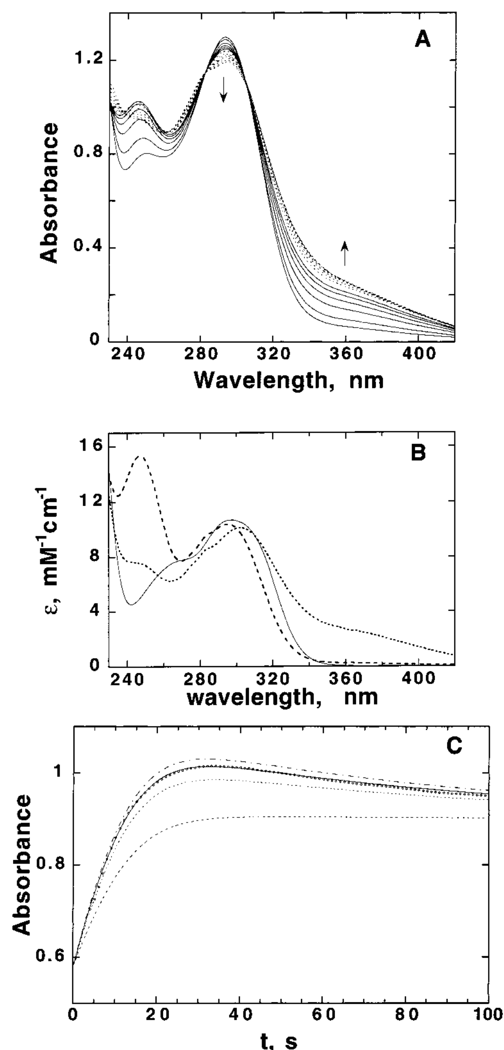

Figure 1. Spectral changes during turnover of 120 μM tetrahydrobiopterin by 10 μM tryptophan hydroxylase in the presence of 650 μM tyrosine at pH 7.0 and 15 °C. (A) Spectra at 5 s intervals are shown for the first 25 s (solid lines) and at 10 s intervals for the next 70 s (dotted lines). (B) Spectra of individual pterin species used to simulate the spectra of A: tetrahydrobiopterin, solid line; hydroxybiopterin, dashed line; quinonoid dihydrobiopterin, dotted line. (C) Comparison of simulated and experimental absorbance traces at 246 nm. The spectral changes were simulated using the mechanism of Scheme 3 with the following values for the rate constants: (—), k_1, 25 000 M^{-1} s^{-1}; k_2, 1 s^{-1}; k_3, 0.30 s^{-1}; k_4, 1.4 s^{-1}; k_5, 0.012 s^{-1}, to yield 18% hydroxypterin; (- - -), k_1, 25 000 M^{-1} s^{-1}; k_2, 1 s^{-1}; k_3, 0.22 s^{-1}; k_4, 1.49 s^{-1}; k_5, 0.012 s^{-1}, to yield 13% hydroxypterin; (- - -), k_1, 25 000 M^{-1} s^{-1}; k_2, 1 s^{-1}; k_3, 0.39 s^{-1}; k_4, 1.31 s^{-1}; k_5, 0.012 s^{-1}, to yield 23% hydroxypterin; (- _ _), k_1, 25 000 M^{-1} s^{-1}; k_2, 1 s^{-1}; k_3, 0.021 s^{-1}; k_4, 1.68 s^{-1}; k_5, 0.012 s^{-1}, to yield 1.2% hydroxypterin.

To determine the amount of hydroxybiopterin formed, the spectral changes were modeled using the mechanism of Scheme 6. Since the concentrations of oxygen and tyrosine are in excess over that of tetrahydrobiopterin, they will not change significantly during the reaction, and only binding of the latter substrate need be considered specifically. Once bound, tetrahydrobiopterin can react to form hydroxybiopterin, detectable at 246 nm, with rate k_3. Alternatively, it can be oxidized directly to quinonoid dihydrobiopterin with rate k_4. Any hydroxybiopterin formed will dehydrate in solution to quinonoid dihydrobiopterin with rate constant k_5. In this model, the relative stoichiometry of hydroxybiopterin formation will be given by $k_3/(k_3 + k_4)$. The ratio of k_2 to k_1 was set to agree with the previously measured K_m value for tetrahydropbioterin.[19] In fitting the data to the model, we found that increasing or decreasing the values of k_1 and k_2 from the final estimates by as little as 2-fold significantly decreased the quality of the fit to the initial phase of the reaction without affecting the total amount of hydroxypterin formed (results not shown). The sum of k_3 and k_4 was kept constant to agree with measured rates of turnover by TRH.[19] The value of k_5 could be measured relatively accurately from the spectral changes at longer times of reaction, since the only reaction occurring then is the dehydration of hydroxybiopterin. The spectra of tetrahydrobiopterin and quinonoid dihydrobiopterin used in the simulations were determined directly from authentic samples of these compounds. The spectrum of 4a-hydroxybiopterin was generated by using the catalytic domain of phenylalanine hydroxylase with phenylalanine as substrate. The spectra of the individual pterins are given in Figure 1B and are similar to those previously described by others.[28] Because of these constraints, selecting the values which gave the best fit was mostly limited to changing the values of k_3 and k_4. Comparison of the absorbance changes at 246 nm with the simulations is given in Figure 1C. The best agreement was achieved when the stoichiometry for hydroxypterin formation was 18% of the tetrahydrobiopterin oxidized, an amount well in excess of the amount of dihydroxyphenylalanine formed in the same experiment. Figure 1C also shows the effects on the predicted spectra of changing this stoichiometry by 5% in either direction or to match the amount of dihydroxyphenylalanine formed. The observed spectral changes at 246 nm are well in excess of those expected if the amounts of hydroxypterin and dihydroxyphenylalanine formed are identical. This result is most consistent with cleavage of the oxygen−oxygen bond to form the hydroxypterin and an additional intermediate prior to oxygen transfer to the amino acid.[29]

Deuterium Kinetic Isotope Effects. As a probe of the reaction of the hydroxylating intermediate with the indole ring of tryptophan, kinetic isotope effects were determined using L-indole-$^2H_5$-tryptophan, L-5-^2H-tryptophan, and L-4-^2H-tryptophan at 295 μM oxygen. 6-Methyltetrahydropterin was used instead of tetrahydrobiopterin because the substrate inhibition which occurs with the latter makes accurate measurement of V_{max} values difficult.[19] The V/K_{trp} value is unaffected by deuteration at any position (results not shown), consistent with irreversible formation of the hydroxylating intermediate prior to amino acid hydroxylation.[11,32] In contrast, there is a significantly inverse effect on the V_{max} value with both L-indole-$^2H_5$-tryptophan and L-5-^2H-tryptophan, but no effect on the V value with L-4-^2H-tryptophan (Table 1). The ^DV value with L-indole-$^2H_5$-tryptophan increases to 0.81 ± 0.03 at 830 μM oxygen.

The mechanisms of Schemes 2 and 3 make different predictions regarding the magnitudes of the secondary isotope

(29) A reviewer has suggested an alternative mechanism in which attack of a water molecule on the terminal oxygen of the peroxypterin results in formation of hydrogen peroxide and the hydroxypterin directly. While we cannot rule this possibility out, we believe it is unlikely. Autoxidation of tetrahydropterins proceeds via formation of the peroxypterin,[30] which then eliminates hydrogen peroxide to form quinonoid dihydropterin (Blair, J. A.; Pearson, A. J. *J. Chem. Soc., Perkin Trans. II* **1974**, 80−88). We have found that this reaction in aqueous solution produces hydrogen peroxide and quinonoid dihydropterin in stoichiometric amounts, with no detectable formation of the hydroxypterin. The very similar autoxidation of reduced flavins similarly proceeds by formation of a 4a-peroxyflavin (Eberlein, G.; Bruice, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 6685−6697); this species also breaks down directly to oxidized flavin and hydrogen peroxide without formation of the 4a-hydroxyflavin in aqueous solution.[31]

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Table 1. Kinetic Isotope Effects for Hydroxylation of Deuterated Tryptophans by Tryptophan Hydroxylase[a]

amino acid	measured DV[b]	equilibrium isotope effect calcd for formation of I[c]	equilibrium isotope effect calcd for formation of II[c]
$^2H_5$-indole−tryptophan	0.91 (0.88, 0.96)[d]	0.889	0.806
5-^{2}H−tryptophan	0.93 (0.84, 0.97)	0.931	0.876
4-^{2}H−tryptophan	1.03 (0.98, 1.10)	1.003	0.873

[a] Conditions: 25 nM tryptophan hydroxylase, 150 μM 6MPH$_4$, 200 mM ammonium sulfate, 3.5 mM dithiothreitol, 12.5 μg/mL catalase, 25 μM ferrous ammonium sulfate, 50 mM MES, pH 7.0, 295 μM oxygen, 15 ^{o}C. [b] The data were fit to eq 2. [c] The structures of the proposed intermediates were calculated using Gaussian 94 with the 6-31G** basis set at the Hartree−Fock level. Equilibrium Isotope effects were calculated using the Bigeleisen equation [25] implemented in QUIVER software. [d] 67% confidence interval.

effects arising from deuteration of the indole ring of tryptophan. The equilibrium isotope effect for a reaction has generally been regarded as the theoretical limit for a secondary kinetic isotope effect,[33] although the kinetic isotope effect can exceed the equilibrium effect under certain conditions.[34] To provide insight into the magnitudes of the expected isotope effects, the equilibrium isotope effects arising upon formation of intermediates **I** and **II** from tryptophan were calculated using ab initio methods. To simplify the calculations, 3-methylindoles were used instead of the complete amino acid. In the calculations of the structure of **I**, the iron atom was replaced with a proton due to the limitations of present levels of ab initio methods in dealing with transition metals.[35] The optimized structures of methylindole, **I** and **II**, used to calculate the isotope effects are given in Figure 2. Table 1 lists the equilibrium isotope effects calculated for formation of **I** and **II** from methylindole. The formation of the cation **I** should result in an inverse isotope effect only upon deuteration of carbon 5. In contrast, the formation of the arene oxide **II** should result in inverse isotope effects with both 4-^{2}H-tryptophan and 5-^{2}H-tryptophan, with a larger effect if both positions are deuterated. Clearly, the observed effects are more consistent with the former prediction.

The NIH Shift. The position and the amount of deuterium in the hydroxytryptophan product when 4-^{2}H-tryptophan or 5-^{2}H-tryptophan is the substrate were used as probes of events occurring after the initial oxygen attack on the indole ring. With 5-^{2}H-tryptophan as substrate, the major product is 4-^{2}H-5-hydroxytryptophan, indicating that an NIH shift has occurred from carbon 5 to carbon 4. With 4-^{2}H-tryptophan as substrate the major product is also 4-^{2}H-5-hydroxytryptophan. The amount of deuterium in the hydroxytryptophan formed from 4-^{2}H- or 5-^{2}H-tryptophan was determined by integration of the ^{1}H NMR spectrum of the respective amino acid product. In the case of 5-^{2}H-tryptophan the hydroxylated product contains 67% deuterium at position 4. In contrast, with 4-^{2}H-tryptophan as substrate 81% of the substrate deuterium at position 4 is retained (Figure 3).

Rate of Product Release. To determine if the rate of product release significantly limits turnover, the rate of hydroxytryptophan formation during the first few turnovers was determined. Enzyme was mixed with saturating concentrations of tryptophan and 6MPH$_4$ in a stopped-flow spectrofluorometer, allowing detection of hydroxytryptophan formation from the increase in fluorescence emission above 305 nm upon excitation at 300. There was a linear increase in product formation from the earliest detection times with no evidence for a burst (Figure 4), indicating that product release does not significantly limit the rate of turnover by TRH.

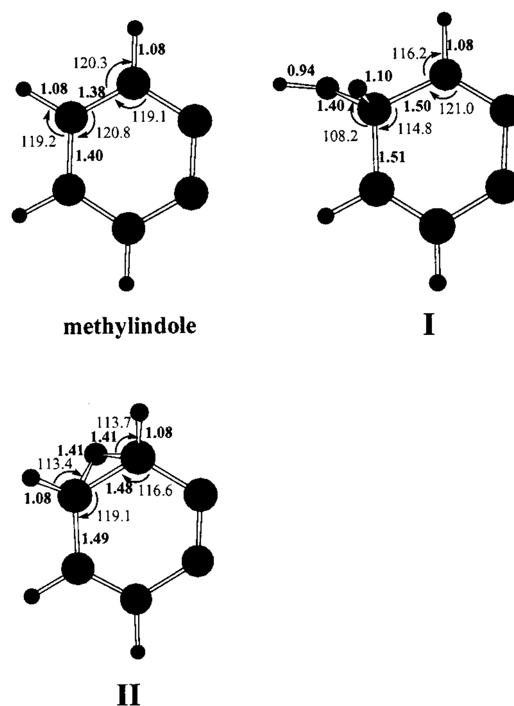

Figure 2. 6-31G* Hartree−Fock optimized structures of intermediates in the hydroxylation of tryptophan. Each calculation was performed on the 3-methylindole analogue. For clarity, only the benzene ring of each structure is shown. The labels correspond to those of Schemes 4 and 5. Distances are given in Å and angles in degrees.

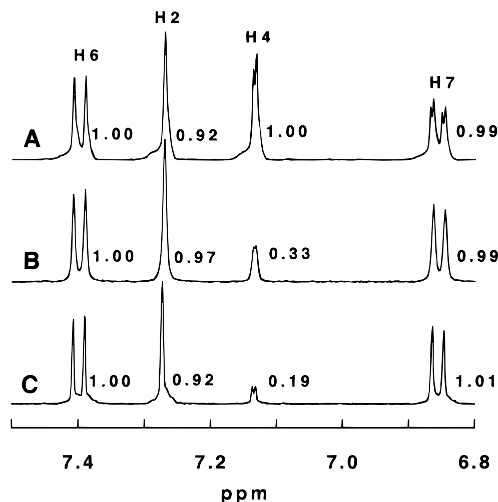

Figure 3. Proton NMR spectra of hydroxytryptophan formed by tryptophan hydroxylase-catalyzed hydroxylation of tryptophan (A), 5-^{2}H-tryptophan (B), or 4-^{2}H-tryptophan (C).

Discussion

Despite the importance of tryptophan hydroxylase as the catalyst for the rate-limiting step in the biosynthesis of the neurotransmitter serotonin, there has been little study of its catalytic mechanism due to the difficulty of obtaining sufficient

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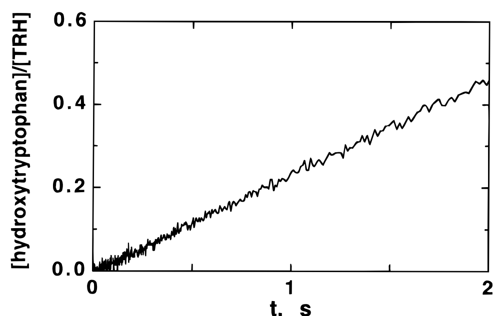

Figure 4. Initial rate of hydroxytryptophan formation during the first turnover. Tryptophan hydroxylase (20 μM) in 400 mM ammonium sulfate, 2 mM dithiothreitol, 100 μg/mL catalase, 50 μM ferrous ammonium sulfate, 100 mM MES, pH 7.0, was mixed in the stopped-flow spectrofluorometer with 120 μM tryptophan, 120 μM 6MPH$_4$, 10 mM HCl, 12 mM dithiothreitol at 15 °C. Formation of hydroxytryptophan was followed by fluorescence, monitoring the emission above 305 nm upon excitation at 300 nm.

amounts of the enzyme. The recent availability of the recombinant catalytic core of the rabbit enzyme has provided a form of tryptophan hydroxylase appropriate for mechanistic studies. The results described here utilizing this recombinant enzyme provide significant new information about the mechanism of this enzyme. In addition, the results extend our mechanistic understanding of the family of pterin-dependent hydroxylases, drawn from studies carried out with the other two members, phenylalanine hydroxylase and tyrosine hydroxylase.

The initial catalytic event in the reaction of these three enzymes must be the formation of the hydroxylating intermediate. The formation of a hydroxypterin as a product of the enzyme-catalyzed reaction,[19] as shown in Scheme 1, establishes that there is a reaction between the pterin and oxygen during catalysis. In the case of phenylalanine hydroxylase, molecular oxygen has been shown to be the source of the oxygen in the hydroxypterin,[36] while the ^{18}O kinetic isotope effects on the tyrosine hydroxylase reaction[37] establish that an early step in catalysis by that enzyme involves a reaction with molecular oxygen. Studies of the reaction of tetrahydropterins with oxygen are consistent with the formation of a hydroxypterin during the autoxidation reaction; decay of this compound would generate dihydropterin and hydrogen peroxide.[30] While no intermediates in the reaction of oxygen with any of the pterin-dependent enzymes have yet been detected directly, a similar formation of a peroxypterin upon the reaction of oxygen and tetrahydropterin as an early step in the catalysis by these enzymes is quite reasonable. Moreover, hydrogen peroxide is produced during the unproductive oxidation of tetrahydropterins by phenylalanine hydroxylase which occurs in the presence of tyrosine[38] in the case of the rat enzyme or in the absence of metal in the case of the bacterial enzyme;[39] this is expected if a peroxypterin breaks down unproductively.

The likelihood of such a peroxypterin as an early intermediate raises the possibility that this species is the actual hydroxylating intermediate in the reaction of the pterin-dependent hydroxylases, including tryptophan hydroxylase. Indeed, in the case of the flavin phenol hydroxylases, an analogous 4a-peroxyflavin is thought to be the hydroxylating intermediate, forming a 4a-hydroxyflavin upon hydroxylation.[31] However, the patterns of products formed from a variety of amino acids as substrates for tyrosine hydroxylase[6] or tryptophan hydroxylase[11] are more similar to what is observed with the cytochrome P450-dependent enzymes. In these systems the hydroxylating intermediate is generally accepted to be a high valence iron-oxo species formed by cleavage of the oxygen−oxygen bond in a ferric peroxide.[40] If a peroxypterin is the hydroxylating intermediate with the pterin-dependent enzymes, cleavage of the oxygen−oxygen bond would be concerted with hydroxylation of the amino acid, as shown in Scheme 4 and as seen with the flavin hydroxylases.[41] In contrast, if a ferryl oxygen is the hydroxylating intermediate, cleavage of the oxygen−oxygen bond would occur prior to hydroxylation of the amino acid, as shown in Scheme 5. In the first case the amount of hydroxypterin and hydroxylated amino acid produced should be identical, while the second could result in an excess of hydroxypterin over hydroxylated amino acid. As shown here, an excess of the hydroxypterin over dihydroxyphenylalanine is produced when tyrosine is utilized as the substrate for tryptophan hydroxylase. This establishes that cleavage of the oxygen−oxygen bond is not concerted with hydroxylation of the amino acid by tryptophan hydroxylase. This result is most consistent with a hydroxylating intermediate which contains a single oxygen atom, as shown in Scheme 5.

Two alternatives have previously been proposed for the mechanism of the subsequent oxygen addition to the amino acid by the pterin-dependent hydroxylases. These are an electrophilic aromatic substitution to form an initial cation, as shown in Scheme 2, and formation of an arene oxide, as shown in Scheme 3. With phenylalanine hydroxylase, an epoxide is the product when L-[2,5-H$_2$]phenylalanine is the substrate, consistent with the involvement of an arene oxide with the normal substrate.[7] In contrast, with tyrosine hydroxylase the product distributions seen with isotopically labeled phenylalanines as substrates are not consistent with the predictions of an arene oxide intermediate.[8] The effects of para-substituents on the products obtained from substituted phenylalanines with the latter enzyme are also more consistent with the mechanism of Scheme 2.[6] The pattern of kinetic isotope effects described here with deuterated tryptophans strongly supports the initial formation of a cationic intermediate rather than an arene oxide. Formation of a cation should result in an inverse deuterium isotope effect if the isotopic substitution is at the site of oxygen addition, but not at the adjacent carbon. In contrast, direct formation of an arene oxide should result in inverse isotope effects at both carbons 4 and 5. This conclusion is qualitatively confirmed by the results of ab initio calculations summarized in Table 1. The possibility of an arene oxide involving carbons 5 and 6 is ruled out by the values obtained with the perdeuterated substrate and by the direction of the NIH shift.[42]

The pattern of isotope effects cannot rule out formation of an arene oxide via a cationic intermediate but only rules out simultaneous formation of bonds to both carbons 4 and 5. While the timing of formation of these bonds has been controversial,

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(42) Because of the assumptions made in calculating the isotope effects, we have made no attempt to correlate the magnitude of the isotope effect with the structure of the transition state. The critical feature of the calculated effects is that formation of **I** should result in an inverse isotope effect only upon deuteration of carbon 5, while formation of **II** will result in inverse effects upon deuteration at either position. Thus, it is the relative magnitudes of the effects at the two positions which is diagnostic of mechanism. The values measured with 295 μM oxygen are clearly not the intrinsic effects, as demonstrated by the more inverse effect seen at 830 μM oxygen.

recent studies of the epoxidation of 1-pentene by *m*-chloroperbenzoic acid are most consistent with a symmetrical transition state for that reaction, in that significantly inverse deuterium kinetic isotope effects are seen at both positions.[43] Even if an arene oxide were to form by collapse of the initial cationic species, rearrangement to the final phenolic product would require that the epoxide ring open to reform the cation prior to the NIH shift.[44] This would place the arene oxide off of the most direct catalytic pathway.

The detection of inverse isotope effects on the V_{max} value with tryptophan hydroxylase establishes that oxygen addition to the aromatic ring occurs in a step which is at least partially rate-limiting. This is consistent with the lack of a burst in product formation and agrees with the conclusion previously drawn from the kinetic parameters of a series of methyl- and azatryptophans as substrates for this enzyme.[11] In contrast, with both tyrosine hydroxylase and phenylalanine hydroxylase, the rate of formation of the hydroxylating intermediate appears to be significantly slower than that of the actual amino acid hydroxylation.[9,10] Both of these enzymes have turnover numbers which are severalfold higher than that of tryptophan hydroxylase. The change in the identity of the rate-limiting step in the case of tryptophan hydroxylase suggests that the decreased rate of turnover is due to a decrease in the rate of hydroxylation rather than the rate of formation of the hydroxylating intermediate.

The observation of an NIH shift in the tryptophan hydroxylase reaction was first made by Renson et al.[4] and has served as the basis for much of the subsequent mechanistic speculation about this enzyme.[5,45] The NMR spectra of Figure 3 confirm that the direction of the shift is from carbon 5 to carbon 4 as initially inferred from the lability of the tritium in the hydroxytryptophan product.[4] In addition, there is a mixture of deuterium and hydrogen at carbon 4, regardless of the initial site of deuteration. The degree of retention of deuterium at carbon 4 is dependent on its origin. Deuterium originating from carbon 5 is twice as likely to be retained in the final product as the hydrogen originally at carbon 4. Conversely, when the deuterium is originally attached to carbon 4, it is 4.3-fold more likely to be retained than the hydrogen originally at carbon 5. This result can occur if hydrogen loss occurs from a symmetrical intermediate containing both hydrogens on carbon 4 in which the loss of the either hydrogen is equally likely, but the loss of one is more sensitive to deuterium substitution. Alternatively, the same products would be seen if cleavage of either of the two carbon−hydrogen bonds in the intermediate results in a deuterium isotope effect of 2.9, but the loss of the hydrogen originally at carbon 4 is only 70% as rapid as the loss of the hydrogen originally at carbon 5. In either case, the relatively small degree of discrimination between the two hydrogens suggests that the loss of a proton to form the phenol either does not involve an active-site base or that both hydrogens on carbon 4 are accessible to the base.

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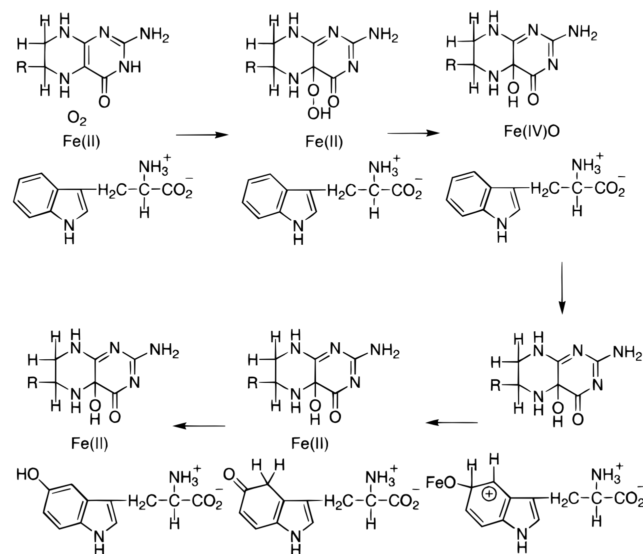
Scheme 7



The results presented in this contribution in combination with previous results allow for the proposal for the catalytic mechanism of tryptophan hydroxylase shown in Scheme 7. The substrates tryptophan, tetrahydrobiopterin, and oxygen bind to the ferrous form of the enzyme. Oxygen initially reacts with the tetrahydropterin in the active site to form a peroxypterin. Heterolytic cleavage of the oxygen−oxygen bond of the peroxypterin results in formation of a ferryl oxo species, the actual hydroxylating intermediate, and the hydroxypterin product. Attack of the activated oxygen species on the aromatic ring of the amino acid substrate generates a cation, which subsequently undergoes an NIH shift. Rearomatization of this species then generates the hydroxylated amino acid product. The results presented here provide evidence for several of these steps.

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