J|A|C|S COMMUNICATIONS

Published on Web 03/26/2002

Intrinsic Deuterium Isotope Effects on Benzylic Hydroxylation by Tyrosine Hydroxylase

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Received January 15, 2002

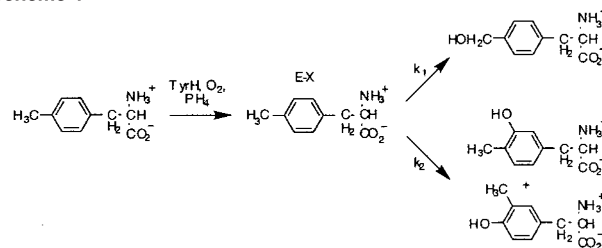
Tyrosine hydroxylase (TyrH) catalyzes the pterin-dependent hydroxylation of tyrosine to dihydroxyphenylalanine.[1-3] This enzyme is a non-heme, mononuclear ferrous iron monooxygenase that belongs to the aromatic amino acid hydroxylase family. This family also includes phenylalanine hydroxylase (PheH) and tryptophan hydroxylase (TrpH). When 4-methylphenylalanine is used as a substrate for TyrH, three products are produced: 3-hydroxy-4-methylphenylalanine, 3-methyl-4-hydroxyphenylalanine, and 4-hydroxymethylphenylalanine.[4] 4-Hydroxymethylphenylalanine results from benzylic hydroxylation as opposed to the aromatic hydroxylation seen with the natural substrate and with the other two products. PheH also catalyzes the benzylic hydroxylation of 4-methylphenylalanine,[5] and TrpH will catalyze the hydroxylation of the methyl group of 5-methyltryptophan.[6] While much work has been done to elucidate the mechanism of aromatic hydroxylation by these enzymes,[1,3,7] the mechanism of benzylic hydroxylation has not been analyzed.

To examine the mechanism of benzylic hydroxylation by TyrH we have determined the intrinsic primary and secondary deuterium isotope effects on this reaction. When 4-methylphenylalanine is used as a substrate for TyrH, 17.7 ± 0.6% of the amino acid products result from benzylic hydroxylation.[8] The relative amount of benzylic hydroxylation decreases as the deuterium content of the substrate methyl group increases (Table 1).[9,10] The effects of isotopic substitution on the relative amounts of the different amino acid products can be analyzed by the mechanism of Scheme 1, where E−X is the hydroxylating species. Here, k_1 is the rate constant for benzylic hydroxylation, and k_2 is the sum of the net rate constants for aromatic hydroxylation at the 3- and 4-positions. The percentage of 4-hydroxymethylphenylalanine produced is $k_1/(k_1 + k_2)$. Since k_1 is the only step that is sensitive to isotopic substitution on the methyl group,[11] the isotope effect on the percent of benzylic hydroxylation is related to the intrinsic isotope effect (${}^{D}k_1 = k_{1H}/k_{1D}$) by eq 1. The value of k_1/k_2 for the nondeuterated substrate is 0.22 ± 0.01. Thus, the intrinsic deuterium isotope effects on benzylic hydroxylation can be calculated for each of the deuterated substrates (Table 1).

$$^{D}(\%\ 4\text{-CH}_2\text{OH-phe}) = \frac{k_{1H}/(k_{1H} + k_2)}{k_{1D}/(k_{1D} + k_2)} = \frac{^{D}k_1 + k_{1H}/k_2}{1 + k_{1H}/k_2} \quad (1)$$

These intrinsic isotope effects are combinations of primary and secondary deuterium isotope effects. When the trideuterated compound is used as a substrate, only a deuterium can be extracted, so that the intrinsic isotope effect is the product of a primary and

Scheme 1



two secondary isotope effects (PS^2). With the dideuterated substrate, removal of a hydrogen exhibits two secondary effects (S^2), while removal of a deuterium exhibits a primary effect and a secondary isotope effect (PS). Likewise, the monodeuterated substrate will show an intrinsic effect equal to a secondary isotope effect when a hydrogen atom is removed and a primary isotope effect when a deuterium is removed. Equations 2 and 3 show the relationships between the isotopic composition of the 4-hydroxymethylphenylalanine product and the primary and secondary isotope effects for each substrate. Here, k is the microscopic rate constant for the abstraction of either a deuterium or a hydrogen atom. The superscript atoms before the dash describe the remaining atoms, and the atom after the dash is the abstracted atom.

$$\frac{\text{4-CDDOH-phe}}{\text{4-CDHOH-phe}} = \frac{k^{\text{DD-H}}}{2k^{\text{HD-D}}} = \frac{1}{2}\left(\frac{k^{\text{DD-H}}}{k^{\text{HH-H}}}\right)\left(\frac{k^{\text{HH-H}}}{k^{\text{HD-D}}}\right) = \frac{P}{2S} \quad (2)$$

$$\frac{\text{4-CDHOH-phe}}{\text{4-CHHOH-phe}} = \frac{2k^{\text{HD-H}}}{k^{\text{HH-D}}} = 2\left(\frac{k^{\text{HD-H}}}{k^{\text{HH-H}}}\right)\left(\frac{k^{\text{HH-H}}}{k^{\text{HH-D}}}\right) = \frac{2P}{S} \quad (3)$$

Using eqs 2 and 3 and the isotope effect of 14.0 for the trideuterated amino acid, the intrinsic primary and secondary deuterium isotope effects were determined from the deuterium content of the hydroxymethylphenylalanine produced from either the monodeuterated or the dideuterated amino acid.[12] These values are given in Table 1. The results from the two different substrates are consistent and give an average intrinsic primary deuterium isotope effect of 9.6 ± 0.9 and an average intrinsic secondary deuterium isotope effect of 1.21 ± 0.08.

The large normal secondary deuterium isotope effect is consistent with significant bond rehybridization at the benzylic carbon during CH bond cleavage;[13] this could arise from a radical or a cationic species.[14] The combination of a large secondary isotope effect and a primary effect greater than the semiclassical limit has previously been interpreted as indicating quantum-mechanical tunneling of the hydrogen.[15] Thus, the present results would add TyrH and the other pterin-dependent hydroxylases to the growing list of enzymes for which tunneling makes a significant contribution to catalysis.

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10.1021/ja025602s CCC: $22.00 © 2002 American Chemical Society

Table 1. Isotope Effects on Benzylic Hydroxylation by Tyrosine Hydroxylase

substrate	% benzylic hydroxylation[a]	intrinsic isotope effect on benzylic hydroxylation	product isotopic ratio[b]	intrinsic primary isotope effect	intrinsic secondary isotope effect
4-C^2H_3-phenylalanine	1.48 ± 0.07	14.0 ± 1.0	-	-	-
4-CH^2H_2-phenylalanine	6.6 ± 1.0	3.0 ± 0.5	4.3 ± 0.4	10.1 ± 0.7	1.18 ± 0.06
4-C $H_2{}^2H$-phenylalanine	12.0 ± 0.8	1.6 ± 0.2	14.5 ± 1.4	9.0 ± 0.6	1.24 ± 0.06

[a] Percent of amino acid product produced by benzylic hydroxylation. [b] Ratio of product due to hydrogen abstraction to the product due to deuterium abstraction as determined by ESI mass spectrometry.

Similarly large primary and secondary deuterium effects have been reported for benzylic hydroxylation by cytochrome P450[16] and dopamine β-monooxygenase.[17] In the case of dopamine β-monooxygenase the hydrogen is proposed to be removed as a hydrogen atom.[17,18] The mechanism of cytochrome P450 is controversial. A mechanism involving hydrogen-atom abstraction had been widely accepted.[19] However, recent analyses of the probable lifetime of the proposed radical by Newcomb and co-workers are consistent with multiple iron species as the hydroxylating intermediates producing both radical and cationic intermediates.[20] Independent of mechanism, in both systems the hydrogen is thought to be abstracted by a metal−oxo species. A high-valence iron−oxo species, Fe(IV)═O, has also been proposed as the hydroxylating intermediate for TyrH.[4] The present results support that proposal and are consistent with the properties of the mononuclear iron site in TyrH resembling those of the heme-based intermediate in cytochrome P450.

Acknowledgment. We thank Dr. Shane Tichy from the LBMS at Texas A&M University for his assistance in acquiring and interpreting the mass spectrometry data. This work was funded by NIH Grants R01-GM47291 (P.F.F.) and T32-GM08523 (P.A.F. and R.P.). The purchase of the ESI mass spectrometer by the LBMS was supported by the Life Sciences Task Force at Texas A&M.

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(8) Standard conditions for determination of the percent of benzylic hydroxylation were 25 mM sodium phosphate, pH 7.0, 1 μM $Fe(II)(NH_4)_2(SO_4)_2$, 1.24 mM amino acid, 10 μM TyrH, 75 μM 6-methyltetrahydropterin. Reactions were run for 2 min at 30 °C in a volume of 100 μL. To quench the reaction, 400 μL of 10 mM sodium borate, pH 9.1, was added, followed by 100 μL 10 mM sodium cyanide and 400 μL 1 mM naphthalene-2,3-dicarboxaldehyde. The reaction was incubated for 20 min at 30 °C. Fifty microliters were then injected onto a Waters C_{18} NovaPak column equilibrated with 12.5 mM sodium phosphate and 0.5% tetrahydrofuran, pH 7.0, and eluted with an acetonitrile gradient. Products were detected by fluorescence with excitation and emission wavelengths of 420 and 490 nm, respectively. Peak areas were quantitated using standard curves from the authentic products.
(9) The deuterated amino acids were synthesized using previously described methods. (a) Reference 4. (b) Lee, Y.; Silverman, R. B. *Org. Lett.* **2000**, *2*, 303−306. Each compound was determined to be greater than 97% deuterated using ^{1}H NMR and mass spectrometry.
(10) When 4-methylphenylalanine is used as a substrate for TyrH, pterin oxidation is partly uncoupled from amino acid hydroxylation, such that the amount of amino acid hydroxylation is substoichiometric to the amount of pterin oxidized.[4] No significant change in coupling could be detected with any of the deuterated substrates.
(11) Isotope effects on binding or on aromatic hydroxylation have not been explicitly ruled out, but any such effects are expected to be much smaller than the values reported here.
(12) Negative ion electrospray mass spectrometry was used to determine the deuterium content of the hydroxymethylphenylalanine. Assays were run under the same conditions as described in ref 4 except for 300 μM 6-methyltetrahydropterin, and products were separated using a water-equilibrated column and an acetonitrile gradient. Fractions containing hydroxymethylphenylalanine were collected and analyzed with negative ion ESI-TOF mass spectrometry. The ratios of the $(m - 1)$ peaks resulting from loss or retention of deuterium were used in the calculations.
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JA025602S