Kinetic Isotope Effects on Hydroxylation of Ring-Deuterated Phenylalanines by Tyrosine Hydroxylase Provide Evidence against Partitioning of an Arene Oxide Intermediate

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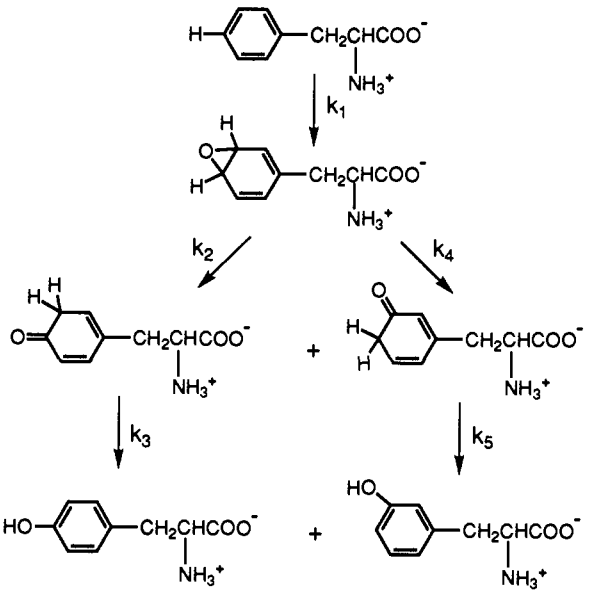
The iron-containing enzymes phenylalanine, tyrosine, and tryptophan hydroxylase constitute a family of tetrahydropterin-dependent aromatic amino acid hydroxylases.[1] While all three are critical in the functioning of the central nervous system, their mechanisms of hydroxylation are poorly understood. When [4-^{3}H]phenylalanine is used as a substrate for either phenylalanine or tyrosine hydroxylase, the tyrosine which is formed retains 70% or 84%, respectively, of the label at the 3-position of the product.[2] On the basis of this result and by analogy with the cytochrome P-450 systems, an arene oxide has been proposed as an intermediate in hydroxylation.[1b,3] Consistent with such a model, Miller and Benkovic[4] have shown that phenylalanine hydroxylase will catalyze the formation of an epoxide from 2,5-dihydrophenylalanine; however, this does not establish whether arene oxide is an obligatory intermediate in tyrosine formation or simply an alternative product formed due to the altered structure of the substrate.

When phenylalanine is used as a substrate for tyrosine hydroxylase, 3-hydroxyphenylalanine is formed in addition to tyrosine,[5] consistent with an arene oxide intermediate which can open to form either substrate (Scheme 1). As a direct test of the obligatory nature of such an arene oxide as an intermediate, the effects of isotopic substitution of the aromatic ring of phenylalanine on product formation have been determined. Since opening of the epoxide in Scheme 1 involves a 1,2 hydrogen shift, it will be subject to a kinetic isotope effect. Consequently, if both hydroxylated products arise from a common arene oxide intermediate, utilization of [4-^{2}H]phenylalanine as substrate should result in a decrease in the amount of tyrosine and a commensurate increase in the amount of 3-hydroxyphenylalanine formed.

Kinetic isotope effects were determined with [4-^{2}H]-, [3,5-$^2H_2$]-, and [2,3,4,5,6-$^2H_5$]phenylalanine[6] as substrates for recombinant rat tyrosine hydroxylase[7] using 6-methyltetrahydropterin as the reducing substrate. The isotope effects were determined in three ways. Isotope effects on the V_{max} values for formation of tyrosine and 3-hydroxyphenylalanine were determined in noncompetitive fashion by measuring the rates of

Scheme 1



formation of each product.[8] The effect of isotopic substitution on the ratio of tyrosine to 3-hydroxyphenylalanine formed was also determined; this is equal to the ratio of steps k_2 and k_4 in Scheme 1. Finally, the amount of hydroxylated products formed per mole of tetrahydropterin consumed was measured.

The results are summarized in Table 1. Deuteration at the position of hydroxylation results in significant kinetic isotope effects on V_{max} values, while no significant β-secondary isotope effects are seen for formation of tyrosine from [3,5-$^2H_2$]-phenylalanine or formation of 3-hydroxyphenylalanine from [4-^{2}H]phenylalanine. The isotope effects for the fully deuterated substrate equal the effects seen upon deuteration of the individual positions, consistent with the lack of β-secondary effects and the independence of the isotope effects at the individual positions on the ring.

The ratio of hydroxylation at the 4-position to that at the 3-position with nondeuterated phenylalanine is 25.5 ± 1.7 at 30 °C. This value is temperature dependent, decreasing with increasing temperature (results not shown). From the effect of temperature on the value of this ratio, a difference of 2.2 kcal/mol in the activation energies for hydroxylation at the two positions can be calculated. The isotope effects on the ratio of hydroxylation at the two positions on the ring are identical to the values determined from the V_{max} values, 1.2 and 1.7 for deuteration at the 4- and 3-positions, respectively (Table 1). The results with perdeuterated phenylalanine again agree with those predicted from the product of the independently measured values.

Oxidation of the tetrahydropterin substrate and formation of hydroxylated phenylalanines are not stoichiometric with rat

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(8) A typical reaction mixture contained 25 mM potassium phosphate, pH 7.0, 75 μg/mL catalase, 2.5 mM phenylalanine, 20 mM 6-methyltetrahydropterin, and 0.4 μM tyrosine hydroxylase in a volume of 0.04 mL at 30 °C. The reaction was initiated by the addition of tetrahydropterin; after 15–180 s, the reaction was terminated by the addition of 25 μL of 0.8 M sodium borate, pH 9.5, and 25 μL of methanol. To this was added 4 μL of a stock solution of phthalaldehyde and 3-mercaptopropionic acid prepared as described by Godel et al. (Godel, H.; Graser, T.; Földi, P.; Pfaender, P.; Fürst, P. *J. Chromatogr.* **1984**, *297*, 49–61). After 3 min, 1.79 mL of 12.5 mM sodium phosphate, pH 7.2, containing 0.5% tetrahydrofuran was added. Precipitated protein was removed by filtering through a 0.2 μM filter; 20 μL was then injected onto a 2 × 150 mm Waters C18 Novapak column. The separation utilized a gradient of 12–16% acetonitrile in 12.5 mM sodium phosphate, pH 7.2, containing 0.5% tetrahydrofuran, with a flow rate of 0.2 mL/min. Derivatized amino acids were detected by fluorescence, with an excitation wavelength of 344 nm and an emission wavelength of 450 nm. To determine the stoichiometry of tetrahydropterin oxidation and hydroxylated amino acids produced, the concentration of tyrosine hydroxylase was increased to about 10 μM and the concentration of 6-methyltetrahydropterin decreased to 50 μM.

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Table 1. Isotope Effects for Hydroxylation of Phenylalanine by Tyrosine Hydroxylase[a]

deuterated phenylalanine	$^{D}V_{tyr}$	$^{D}V_{3HOphe}$	$^{D}p/m^{b}$	$^{D}coupling^{c}$
4-^{2}H	1.22 ±0.01[d]	1.01 ±0.09	1.22 ±0.02	1.21 ±0.07
3,5-$^{2}H_2$	1.04 ±0.07	1.72 ±0.28	0.55 ±0.03	1.05 ±0.05
$^{2}H^{5}$	1.21 ±0.09	1.52 ±0.21	0.77 ±0.13	1.29 ±0.12

[a] Isotope effects were determined in 25 mM potassium phosphate, 75 μg/mL catalase, 2.5 mM phenylalanine, pH 7.0, at 30 °C. [b] Isotope effect on the ratio of tyrosine to 3-hydroxyphenylalanine produced. [c] Isotope effect on the ratio of hydroxylated phenylalanines produced to 6-methyltetrahydropterin consumed. [d] Standard error.

tyrosine hydroxylase. Instead, a significant amount of reducing equivalents are discharged unproductively. With nondeuterated phenylalanine, 20.7 ± 0.7% of the 6-methyltetrahydropterin consumed results in hydroxylated products. The effects of isotope substitution on this value are identical to those on the rate of formation of tyrosine and on the ratio of the two products (Table 1). This is again consistent with a decrease in the amount of tyrosine formation upon deuteration of the 4-position of phenylalanine without a commensurate increase in the amount of hydroxylation at the 3-position.

All three methods of measuring the isotope effects for phenylalanine hydroxylation are consistent with the independence of the values for formation of tyrosine and 3-hydroxyphenylalanine. Substitution at the 4-position results in an isotope effect of 1.2 on the rate of tyrosine formation, with no significant effect on the rate of formation of 3-hydroxyphenylalanine. Similarly, substitution at the 3- and 5-positions results in an isotope effect of about 1.7 on 3-hydroxyphenylalanine formation, but no change in the rate of tyrosine formation. Clearly, there is no increase in hydroxylation at the nondeuterated position to balance the decrease at the isotopically substituted position as predicted if an arene oxide intermediate is partitioning between the two hydroxylated products. This is most clearly seen by the lack of an increase in 3-hydroxyphenylalanine formation with [4-^{2}H]-phenylalanine, where a decrease in tyrosine formation of $1 - (^{1}/_{1.2})$ or 17% would result in an increase in 3-hydroxyphenylalanine formation of 5-fold if such partitioning were occurring. Thus, these results are not those predicted for partitioning of an arene oxide to form either 3-hydroxyphenylalanine or tyrosine.[9] Instead, they suggest that partitioning occurs upon initial attack of the activated oxygen species on the substrate. Indeed, even in the case of hydroxylation of substituted benzenes by cytochrome P-450, evidence that a significant fraction of hydroxylation also occurs without formation of an arene oxide has been found by several approaches.[10]

While the qualitative interpretation of the data is clear, quantitative analysis reveals some unexpected trends. Initial attack of an activated oxygen on the aromatic ring of the substrate would be expected to result in an inverse secondary kinetic isotope effect with a value as small as 0.8.[11] Normal primary kinetic isotope effects would be expected to be associated with subsequent 1,2 hydride transfer and rearomatization. Such isotope effects would be expected on the V_{max} values, since that kinetic parameter contains the rates of all of the first-order steps.[12] However, the ratio of hydroxylation at the two positions will be determined by the relative rates of oxygen addition.[13] A normal isotope effect on this value will be seen only if oxygen attack is coupled in some fashion to cleavage of the CH bond. This could occur if the transition state for oxygen attack also involves some transfer of the hydrogen or if the initial attack of the activated oxygen is reversible.[14] In either case, a small inverse secondary effect would be masked by a larger primary effect. Formation of a metallaoxetane intermediate would also account for these results, but the involvement of metallaoxetanes in hydroxylations involving metalloproteins is still controversial.[15]

Despite this last point, the results presented here are clearly inconsistent with partitioning of an arene oxide as an obligatory intermediate in the hydroxylation of phenylalanine by tyrosine hydroxylase. They also suggest that hydroxylations of the normal substrates by this entire group of enzymes do not involve arene oxide intermediates.

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(9) Small inverse isotope effects are predicted for hydroxylation at the nondeuterated position if partitioning occurs upon the initial attack of oxygen. Thus, a decrease in tyrosine formation of 1.2-fold must be partitioned among hydroxylation at the 3-position and the uncoupled reaction. Since uncoupling occurs 125 times as often as 3-hydroxylation, an isotope effect of 0.996 on 3-hydroxyphenylalanine formation is predicted. This would not be detectable. Similarly, the decreased formation of 3-hydroxyphenylalanine upon deuteration at the 3- and 5-positions will result in an increase of tyrosine formation of only 1.005.

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(12) When tyrosine is used as substrate for tyrosine hydroxylase, product release is rapid relative to previous chemical steps (Fitzpatrick, P. F. *Biochemistry* **1991**, *30*, 3658–3662).

(13) An alternative explanation is that there are two forms of the enzyme, one of which hydroxylates at the 3-position and one at the 4-position of phenylalanine. If these cannot interconvert after substrate binds, the independent isotope effects would be observed. However, in our hands the ratio of hydroxylation at the two positions is independent of the individual preparation of the enzyme, whether it is expressed in baculovirus or bacteria, or whether it is phosphorylated.

(14) No exchange of label from substrate in to the solvent was detected when the reaction was run in [^{3}H]H_2O (12.5 mCi/mL) and the remaining phenylalanine isolated.

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