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A Mechanism for Hydroxylation by Tyrosine Hydroxylase Based on Partitioning of Substituted Phenylalanines†

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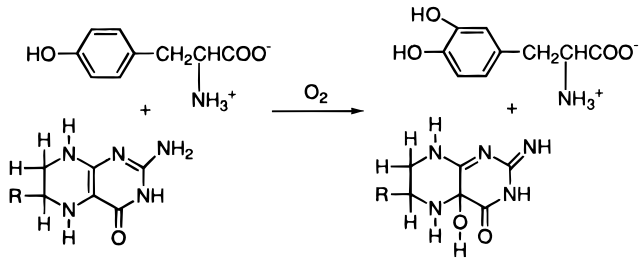
ABSTRACT: The iron-containing enzyme tyrosine hydroxylase catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine. A series of 4-X-substituted (X = H, F, Br, Cl, CH_3, or CH_3O) phenylalanines have been characterized as substrates to gain insight into the mechanism of hydroxylation. Multiple hydroxylated products were formed in most cases. As the size of the substituent at the 4-position increased, the site of hydroxylation switched from the 4- to the 3-position of the aromatic ring. The total amount of product formed with each amino acid showed a very good correlation with the σ parameter of the substituent, with ρ values of -4.3 ± 0.7 or -5.6 ± 0.8 when tetrahydrobiopterin or 6-methyltetrahydropterin, respectively, was used as cosubstrate. These values are consistent with a highly electron deficient transition state for hydroxylation. Oxygen addition at the 4-position resulted in either elimination of the substituent to form tyrosine or an NIH shift to form the respective 3-X-tyrosine. The relative amount of the product due to an NIH shift decreased in the order Br > CH_3 > Cl ≫ F ∼ CH_3O ∼ 0. A chemical mechanism for hydroxylation by tyrosine hydroxylase is presented to account for product formation from the various 4-substituted phenylalanines.

Tyrosine hydroxylase is a non-heme iron-containing enzyme that catalyzes the conversion of tyrosine to dihydroxyphenylalanine (dopa)[1] (Kaufman & Kaufman, 1985). The other substrates for the reaction are molecular oxygen and tetrahydrobiopterin, with a labile 4a-hydroxytetrahydropterin as the other product (Scheme 1) (Haavik & Flatmark, 1987). This hydroxylation is the first and rate-limiting step in the biosynthesis of the catecholamine neurotransmitters dopamine, epinephrine, and norepinephrine. Tyrosine hydroxylase belongs to a family of pterin-dependent amino acid hydroxylases which also includes phenylalanine hydroxylase and tryptophan hydroxylase. Phenylalanine hydroxylase is involved in phenylalanine catabolism, and tryptophan hydroxylase catalyzes the first step in the bio-

Scheme 1



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[1] Abbreviations: dopa, dihydroxyphenylalanine; $6MPH_4$, 6-methyltetrahydropterin; BH_4, tetrahydrobiopterin.

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synthesis of the neurotransmitter serotonin.

The chemical mechanisms of these enzymes are poorly understood. Studies of the kinetics of tyrosine hydroxylase are consistent with the rate-limiting step being formation of the oxygenating intermediate rather than hydroxylation of the amino acid. Thus, while chemical steps are rate-limiting (Fitzpatrick, 1991b), V_{max} values are invariant with substituted phenylalanines of very different intrinsic reactivity and no primary or solvent kinetic isotope effects are seen with tyrosine as substrate (Fitzpatrick, 1991a,b). Several mechanisms for the hydroxylation step have been proposed over the years. Electrophilic aromatic substitution of the phenolate form of the substrate by a tetrahydropterin hydroperoxide, in analogy to the flavoprotein hydroxylases (Ballou, 1982), is not consistent with results with transition-state analogs (Fitzpatrick, 1991a). When phenylalanine is a substrate for tyrosine hydroxylase, both 3-hydroxyphenylalanine and tyrosine are formed (Tong et al., 1971; Fukami et al., 1990). This would be consistent with partitioning of an arene oxide intermediate. Indeed, Miller and Benkovic (1988) have shown that phenylalanine hydroxylase can form an epoxide from dihydrophenylalanine. However, an arene oxide has been ruled out as an intermediate in the hydroxylation of phenylalanine by tyrosine hydroxylase by use of isotopically labeled phenylalanines (Fitzpatrick, 1994). The results of that study were consistent with direct attack of an oxygenating species at either the 4- or the 3-position of the substrate.

Since the rate-limiting step in turnover appears to be formation of the hydroxylating intermediate rather than hydroxylation of the amino acid substrate, standard kinetic analyses are unlikely to provide insight into the mechanism of hydroxylation. Instead, we have adopted a strategy which focuses on events after the hydroxylating species forms. Multiple hydroxylated products have been reported with some nonphysiological substrates for tyrosine hydroxylase (Kaufman & Kaufman, 1985). In addition, oxidation of the tetrahydropterin substrate can be uncoupled from hydroxylation of the amino acid under some conditions (Kaufman & Kaufman, 1985). This suggests that there are multiple mechanistic pathways available to the hydroxylating intermediate once it forms. We have characterized the partitioning among these pathways with a series of substituted phenylalanines to probe the mechanism of hydroxylation by tyrosine hydroxylase. The results of these studies are reported here.

EXPERIMENTAL PROCEDURES

Materials. Tyrosine, phenylalanine, 4-aminophenylalanine, 4-methoxyphenylalanine, 4-fluorophenylalanine, 4-chlorophenylalanine, 4-bromophenylalanine, 3-chlorotyrosine, 3-iodotyrosine, sheep liver dihydropteridine reductase, and NADH were from Sigma Chemical Co., St. Louis, MO. 4-Methoxyphenylalanine, 3-chlorotyrosine, and 3-iodotyrosine were further purified using a 19 × 300 mm Waters C18 μBondapak column in 6% acetonitrile. 3-Fluorotyrosine, 3-mercaptopropionic acid, and all chemicals used for organic synthesis were from Aldrich Chemical Co., Milwaukee, WI. *m*-Tyrosine was from Lancaster Syntheses, Inc., Windham, NH. *o*-Phthalaldehyde was from Fluka Chemical Corp., Ronkonkoma, NY. Tetrahydrobiopterin was from Research Biochemicals International, Natick, MA. 6-Methyltetrahydropterin was synthesized as described previously (Fitzpatrick, 1988).

Recombinant rat tyrosine hydroxylase was purified as described previously (Daubner et al., 1992). The enzyme concentration was determined using an $\epsilon_{280}^{1\%}$ value of 10.4 (Haavik et al., 1988) and a subunit molecular weight of 56000. Catalase was from Boehringer Mannheim, Indianapolis, IN.

Methods. UV/visible spectra were taken on a Hewlett-Packard Model 8542A diode array spectrophotometer. Steady-state kinetic measurements were taken on a Gilford 260 spectrophotometer with a Gilford 6051 chart recorder as described previously (Fitzpatrick, 1991b). Variable amounts of substrate were added to a solution of 0.6 unit/mL sheep dihydropteridine reductase, 200 μM NADH, 100 μg/mL catalase, and 50 mM HEPES (pH 7.1) for a total volume of 0.7 mL. The assay mixture was incubated at 25 °C for 3 min, and either 6-methyltetrahydropterin or tetrahydrobiopterin was added. After 1−2 min, tyrosine hydroxylase was injected. The initial velocities were calculated by subtracting the tetrahydropterin autooxidation rate determined from the portion of the reaction in the absence of enzyme from the enzymatic rate. The resulting steady-state data were fit to eq 1 using the KinetAsyst software from IntelliKinetics, State College, PA.

$$v = \frac{VA}{K_{aa} + A} \tag{1}$$

Product analyses were performed by HPLC using a Waters 625LC pump with a Waters automated gradient controller, a Waters 470 scanning fluorescence detector, and a Spectra-Physics 4400 integrator as described previously (Fitzpatrick, 1994). A reaction mixture containing 25 mM potassium phosphate (pH 7.0), 75 μg/mL catalase, amino acid substrate, 500 μM tetrahydropterin, and 1.0 μM tyrosine hydroxylase in a volume of 0.04 mL was incubated at 30 °C for 30 s. The reaction was initiated by the addition of the tetrahydropterin. The reaction was quenched by the addition of 25 μL of 0.8 M sodium borate (pH 9.5) and 25 μL of methanol. The solution was derivatized with 4 μL of a solution of 10 mg/mL phthalaldehyde and 1% 3-mercaptopropionic acid in 1 M sodium borate (pH 9.5) and 10% methanol. After 3 min, 1.79 mL of 12.5 mM sodium phosphate (pH 7.2) and 0.5% tetrahydrofuran, was added. The solution was filtered through a 0.2 μm filter, and 20 μL was then injected onto a 2 × 150 mm Waters C18 Novapak column. Product separation was achieved using a 10 to 22% gradient of acetonitrile in 12.5 mM sodium phosphate (pH 7.2) containing 0.5% tetrahydrofuran, with a flow rate of 0.2 mL/min. The 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 tetrahydropterin was decreased to 100 μM.

Tetrahydrobiopterin and 6-methyltetrahydropterin concentrations were determined in 2 M perchloric acid using ϵ_{266} values of 18 mM^{-1} cm^{-1} (Hill et al., 1988) and 17.8 mM^{-1} cm^{-1} (Shiman et al., 1971), respectively. Tetrahydropterin stocks were made up fresh daily.

Table 1: Apparent Steady-State Kinetic Parameters for Alternate Substrates of Tyrosine Hydroxylase at pH 7.1 and 25 °C[a]

substrate	K_{aa} (μM)	V_{max} (min^{-1})
phenylalanine	41.6 ± 9.1	16.9 ± 1.2
tyrosine	4.9 ± 1.9	36.9 ± 4.1
4-methoxyphenylalanine	1350 ± 290	20.0 ± 2.2
4-methylphenylalanine	1100 ± 200	19.3 ± 1.9
4-fluorophenylalanine	110 ± 20	48.2 ± 4.3
4-chlorophenylalanine	220 ± 50	13.8 ± 1.1
4-bromophenylalanine	240 ± 60	9.2 ± 0.9

[a] Initial velocities were determined by following the rate of dihydropterin production with variable amounts of amino acid, 10 μM 6-methyltetrahydropterin, and 0.6 unit/mL sheep dihydropterin reductase, 200 μM NADH, 100 μg/mL catalase, 0.25 μM tyrosine hydroxylase, and 50 mM HEPES (pH 7.1) in a volume of 0.7 mL at 25 °C.

RESULTS

Steady-State Kinetics with Substituted Phenylalanines. Steady-state kinetic parameters were determined with several 4-substituted phenylalanines as substrates for tyrosine hydroxylase using 6-methyltetrahydropterin as the cosubstrate. The concentration of the tetrahydropterin was maintained below the K_m value of around 30 μM to decrease the background rate due to tetrahydropterin autooxidation. This resulted in apparent parameters since all substrates were not at saturating levels. The kinetic parameters with the 4-substituted phenylalanines are summarized in Table 1. The results indicate that these amino acids are all substrates for the enzyme. There was a relatively slight change in the apparent V_{max} value over the range of substrates used. This suggests that the slow step in catalysis does not involve the aromatic amino acid, as has been shown from more extensive kinetic analyses with both the rat and bovine enzymes (Fitzpatrick, 1991a; Meyer & Fitzpatrick, 1992). The apparent K_m values increase with increasing size of the substituent at the C-4 position of the aromatic ring. A similar result was found previously using bovine tyrosine hydroxylase (Meyer & Fitzpatrick, 1992).

Product Formation from Substituted Phenylalanines. The products formed from the substituted phenylalanines were determined by HPLC using synthesized standards. Figure 1 shows representative time courses for product formation from 4-fluorophenylalanine (panel A) and 4-chlorophenylalanine (panel B). dopa was formed as a product from both substrates. However, there was a lag in dopa formation, in contrast to the rate of formation of either tyrosine or 4-chloro-3-hydroxyphenylalanine. This suggested that dopa was not an initial product of the enzyme. The dopa formed could be from two sources: (1) further hydroxylation of tyrosine formed as an initial product by halogen elimination (pathway 3 in Scheme 2), or (2) further hydroxylation of the initial halogenated, hydroxylated product (pathways 1 and 2). To test the latter possibility, halogenated, hydroxylated phenylalanines were tested directly as substrates and any amino acid products were then identified by HPLC of the reaction mixtures. dopa was formed as the only amino acid product from 4-chloro-3-hydroxyphenylalanine, 4-bromo-3-hydroxyphenylalanine (pathway 5 in Scheme 2), and 3-fluoro-4-hydroxyphenylalanine (pathway 4). In contrast, dopa was not formed from either 3-chloro-4-hydroxyphenylalanine or 3-bromo-4-hydroxyphenylalanine (pathway 4). These results are summarized in Table 2. For further studies, reaction times were kept at 60 s and the initial amino acid concentration was increased to keep dopa formation low and to facilitate quantitation of the initial products from the enzyme-catalyzed reaction.

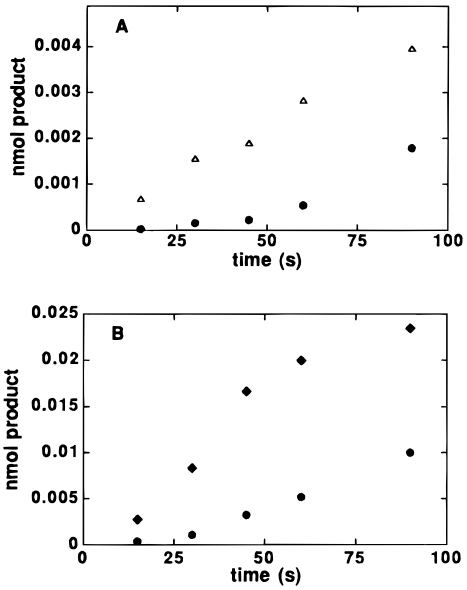
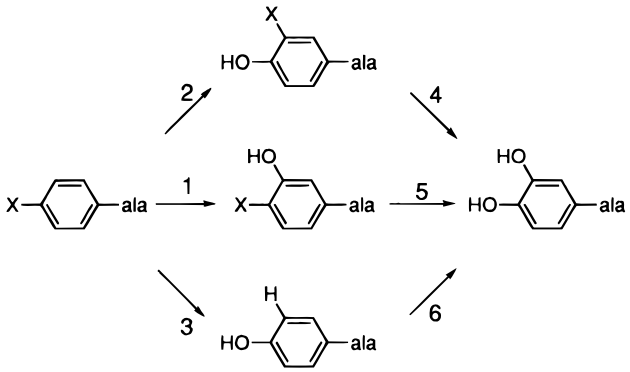


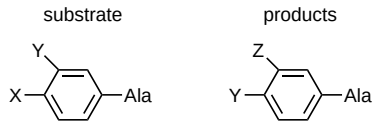
FIGURE 1: Product formation from 4-fluorophenylalanine (A) and 4-chlorophenylalanine (B) as substrates for tyrosine hydroxylase. Data points represent the amount of dopa (●), tyrosine (△), or 4-chloro-3-hydroxyphenylalanine (◆) formed at specified time intervals. For clarity, the 4-chlorophenylalanine products 3-chloro-4-hydroxyphenylalanine and tyrosine are not shown in panel B. Conditions were as follows: 25 mM potassium phosphate (pH 7.0), 0.625 mM amino acid, 100 μg/mL catalase, 500 μM 6-methyltetrahydropterin, and 1.0 μM tyrosine hydroxylase in a volume of 0.04 mL at 30 °C. Reactions were run as described in Table 2.

Scheme 2



Multiple product formation was observed for many of the 4-substituted phenylalanines as substrates for tyrosine hydroxylase (Table 2). When 4-bromophenylalanine was used as a substrate, 4-bromo-3-hydroxyphenylalanine, 3-bromo-4-hydroxyphenylalanine, and tyrosine were all formed as initial products (paths 1−3 in Scheme 2). With 4-chlorophenylalanine as a substrate, similar products were detected: 4-chloro-3-hydroxyphenylalanine, 3-chloro-4-hydroxyphenylalanine, and tyrosine. The only product found for 4-fluorophenylalanine as a substrate was tyrosine (pathway 3). Multiple products were also found with 4-methylphenylalanine as a substrate. Three products were detected: 4-methyl-3-hydroxyphenylalanine (pathway 1), 3-methyl-4-hydroxyphenylalanine (pathway 2), and 4-(hydroxymethyl)phenylalanine. Hydroxylation of phenylalanine produced two products, tyrosine and 3-hydroxyphenylalanine, as has been reported previously (Fitzpatrick, 1994). 4-Meth-

Table 2: Products from Alternate Substrates of Tyrosine Hydroxylase[a]



X	Y	Y	Z	% formation	% substrate coupling to BH_4[b]	% substrate coupling to $6MPH_4$[b]	NIH shift[c]	para versus meta addition[d]
H	H	H	OH	4[e]	27.0 ± 1.1	26.1 ± 3.0	0.84[f]	24:1[e]
		HO	H	96[e]				
CH_3	H	CH_3	HO	51 ± 1	70.7 ± 7.3	61.4 ± 9.0	0.41	0.41:1
		HO	CH_3	21 ± 1				
		$HOCH_2$	H	28 ± 1				
CH_3O	H	CH_3O	HO	100	88.4 ± 0.8	97.6 ± 1.1	0	<1:100
		HO	CH_3O	<1				
F	H	F	HO	<1	34.5 ± 10.2	27.0 ± 4.4	0	>99:1
		HO	F	<1				
		HO	H	100				
Cl	H	Cl	HO	51 ± 6	10.5 ± 1.5	5.0 ± 0.8	0.22	0.96:1
		HO	Cl	11 ± 3				
		HO	H	38 ± 3				
Br	H	Br	HO	40 ± 9	2.1 ± 0.4	1.4 ± 0.8	0.61	1.5:1
		HO	Br	35 ± 12				
		HO	H	25 ± 2				
HO[g]	F	HO	HO	100	20			
HO[g]	Cl	HO	HO	<1	<1			
Cl[g]	HO	HO	HO	100	6			
HO[g]	Br	HO	HO	<1	<1			
Br[g]	HO	HO	HO	100	46			

[a] Conditions: 25 mM potassium phosphate (pH 7.0), 1−2 mM amino acid, 100 μg/mL catalase, 100 μM tetrahydropterin, and 10 μM tyrosine hydroxylase in a volume of 0.04 mL. Reaction mixtures were incubated for 30 s at 30 °C and reactions initiated by addition of the tetrahydropterin. Reactions were quenched after 1 min with 25 μL of 0.8 M sodium borate (pH 9.5) and 25 μL of methanol. The products were identified by HPLC as described in Experimental Procedures. [b] Coupling is calculated by dividing the total nanomoles of hydroxylated amino acid formed by the nanomoles of tetrahydropterin consumed. [c] Nanomoles of 3-X-4-OH-Phe/(nanomoles of tyrosine + nanomoles of 3-X-4-OH-phe). [d] (Nanomoles of tyrosine + nanomoles of 4-OH-3-X-Phe)/nanomoles of 3-OH-4-X-phe. [e] Fitzpatrick (1994). [f] Daly et al. (1968) for [4-^{3}H]phenylalanine. [g] 20 nmol of $6MPH_4$ used in reaction.

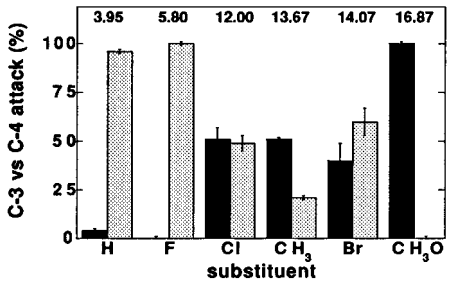


FIGURE 2: Effect of increasing van der Waals volume of the C-4 group of the substituted phenylalanine on the relative amount of attack at either the C-3 position (black) or the C-4 position (stippled). Product formation data were taken from Table 2. Volumes of the substituents shown at the top of the figure in cm^3/mol are from Bondi (1964).

oxyphenylalanine gave a single product; only 4-methoxy-3-hydroxyphenylalanine was detected (pathway 1).

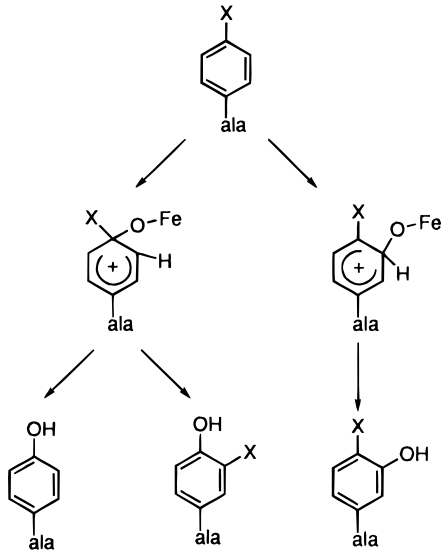
The product distributions summarized in Table 2 show a variation in the sites of hydroxylation with different substrates. As shown in Figure 2, as the size of the substituent at the 4-position of the aromatic ring increases, there is an increase in the proportion of substrate which has been hydroxylated at the 3-position relative to that hydroxylated at the 4-position. In other words, the larger substituents promote hydroxylation at the C-3 position over that at the C-4 position. These results suggest that sterics are a primary determinant of the initial site of attack of the oxygenating intermediate on the amino acid substrate. Not included in this analysis is the formation of 4-(hydroxymethyl)phenylalanine from 4-methyl phenylalanine, since hydroxylation in this case is not at the aromatic ring.

Stoichiometry of Hydroxylation. With tyrosine as substrate, the stoichiometry of the tyrosine hydroxylase reaction is as shown in Scheme 1 (Kaufman & Kaufman, 1985). For each mole of tetrahydrobiopterin oxidized, one mole of dopa is formed. However, when nonphysiological substrates are used, a fraction of the tetrahydropterin can be oxidized without concomitant formation of hydroxylated amino acid, as is the case with many monooxygenases. Coupling can be defined as the ratio of amino acid hydroxylation to tetrahydropterin oxidation by tyrosine hydroxylase. The amount of coupling was determined for the various 4-substituted phenylalanines listed in Table 2. The coupling was measured by adding a known, limiting amount of tetrahydropterin to the enzymatic reaction mixture and running the reaction until all the tetrahydropterin had been consumed. The amount of product formation was then quantitated by HPLC using synthesized standards. This was done using both 6-methyltetrahydropterin and tetrahydrobiopterin as the reducing substrate. The results were independent of the time of reaction and the amount of tyrosine hydroxylase added. The couplings of tetrahydropterin oxidation and substrate hydroxylation for the various substrates for tyrosine hydroxylase are shown in Table 2. There was a wide range of couplings observed, from very little with 4-bromophenylalanine as a substrate to nearly complete coupling with 4-methoxyphenylalanine.

DISCUSSION

The rate-limiting step in the tyrosine hydroxylase reaction appears to be formation of the hydroxylating intermediate.

Scheme 3



Burst experiments have established that all steps after oxygen addition to the amino acid are rapid (Fitzpatrick, 1991b). The V_{max} value is invariant with substrates of greatly different reactivity (Fitzpatrick, 1991a), consistent with a slow step in the reaction which does not involve the amino acid. There is a significant $^{18}V/K$ isotope effect with O_2, directly demonstrating a change in the bond order to oxygen in a rate-determining transition state.[2] All of these results are consistent with relatively slow and irreversible formation of the oxygenating intermediate followed by the much more rapid hydroxylation of the substrate. Consequently, measurements of kinetic parameters with amino acids of varying reactivity yield little information regarding the mechanism of hydroxylation. To circumvent the limitations of kinetic analyses, we have adopted a strategy of analyzing the partitioning of alternate substrates among multiple products. Different hydroxylated products could arise from a single substrate either due to differences in the site of initial attack of the activated oxygen species on the substrate or due to alternative chemical rearrangements which the initial intermediate undergoes. In either case, the multiple products arise because of events which occur after formation of the hydroxylating intermediate. Thus, product distributions can be used as probes of the mechanism of hydroxylation.

The data presented here are consistent with the mechanism for tyrosine hydroxylase shown in Scheme 3. Formation of different hydroxylated products can be attributed to partitioning of intermediates at different branch points in this overall mechanism.

With the substrates examined here, not all of the reducing equivalents from the tetrahydropterin were used to form hydroxylated amino acid. This uncoupling of reductant oxidation and substrate hydroxylation is a frequent feature of monooxygenases when they are acting on substrates other than the physiological substrate (Gorsky et al., 1984; Shiman, 1985). The degree of coupling of tetrahydropterin oxidation and amino acid hydroxylation can be used as a probe of the mechanism of hydroxylation. Partial uncoupling must be due to partitioning of a hydroxylating intermediate between

[2] P. F. Fitzpatrick, W. Francisco, G. Tian, and J. P. Klinman, unpublished observations.

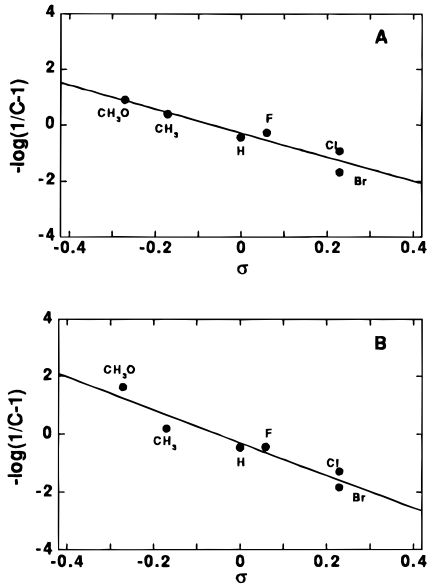
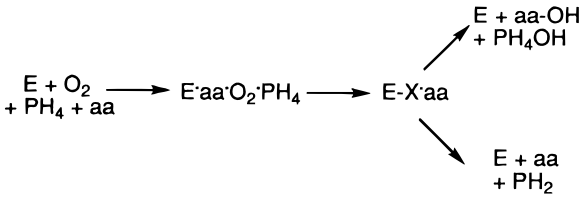


FIGURE 3: Hammett plots of coupling of tetrahydropterin oxidation to amino acid hydroxylation for substrates for tyrosine hydroxylase with BH_4 (A) or $6MPH_4$ (B). The lines are fits of the equation $-\log(1/C-1) = \rho\sigma + b$.

Scheme 4



productive and unproductive pathways (Scheme 4). The amount of coupling would be determined by the relative rates of hydroxylation by (k_{hyd}) and unproductive breakdown of (k_{un}) the hydroxylating intermediate E−X. It is reasonable to assume k_{un} is relatively insensitive to the structure of the bound amino acid substrate, given the constant V_{max} values for many of these substrates (Fitzpatrick, 1991a) and the identical ^{18}O isotope effects seen with both coupled and uncoupled substrates.[2] For the mechanism in Scheme 4, the coupling *C*, the number of moles of hydroxylated amino acid formed per mole of tetrahydropterin oxidized, is given by eq 2. Equation 2 can readily be rearranged to eq 3, which gives an explicit relationship between the measured coupling and the relative rate of attack of the intermediate on the amino acid. Figure 3 shows plots of the values of $-\log(1/C-1)$ as a function of the σ values of the substituents on the aromatic rings of the amino acid substrates. With both tetrahydrobiopterin and 6-methyltetrahydropterin, the value of $-\log(1/C-1)$ correlates well with the electronic contribution of the substituent. Fits of comparable quality were obtained with both σ and σ^+ (Table 3). The ρ values determined with tetrahydrobiopterin varied slightly from the ρ values determined with 6-methyltetrahydropterin, but both are large and negative.

$$C = \frac{k_{hyd}}{k_{hyd} + k_{un}} \quad (2)$$

$$-\log(1/C-1) = \log(k_{hyd}) - \log(k_{un}) \quad (3)$$

These ρ values of approximately −5 are consistent with a very electron deficient transition state for the attack of the

Table 3: Hammett Constants Determined from Coupling for Substrates of Tyrosine Hydroxylase[a]

	BH_4		$6MPH_4$	
	ρ	R	ρ	R^b
σ	-4.3 ± 0.7	0.95	-5.6 ± 0.8	0.96
σ^+	-4.6 ± 0.4	0.98	-5.9 ± 0.8	0.97
σ^-	-2.4 ± 0.5	0.91	-3.3 ± 0.4	0.97

[a] ρ values determined from coupling values from Table 2 for amino acid substrates using the equation $-\log(1/C-1)$, where C is the coupling. [b] R is the correlation coefficient from the fit of the data to the equation $-\log(1/C-1) = \rho\sigma + b$.

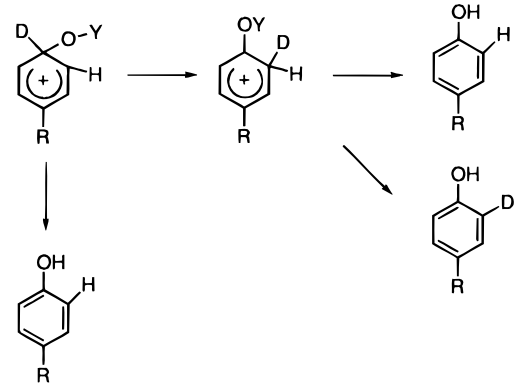
oxygenating intermediate on the amino acid substrate. The most straightforward rationale for such a transition state is that attack of the hydroxylating intermediate generates a cationic intermediate.[3] Another possibility is the formation of a neutral radical intermediate by attack of an extremely electrophilic hydroxylating species. However, the expected ρ value for the latter reaction is in the range of 0.5 to -1.0 (Pryor et al., 1973).

The proposed cation can form by attack at either the 3- or the 4-position of the aromatic ring. Previous results with deuterated phenylalanines were consistent with partitioning between attack at these two sites occurring at the time of formation of the new bond to oxygen (Fitzpatrick, 1994). The more extensive analyses described here are also consistent with such a conclusion. The data summarized in Figure 2 suggest that the site of initial attack is determined primarily by steric factors. The increasing K_m values for the amino acid substrates as the volume of the para substituent of the substrate increases are consistent with a restricted active site in the area of this position. In addition, with bovine tyrosine hydroxylase, Meyer and Fitzpatrick (1992) found that there was a linear correlation between the volume of the substituent at the 4-position and the K_m value for the amino acid substrate.

Collapse of the cationic species would result in formation of the phenolic products. When [4-^{3}H]phenylalanine is used as substrate for tyrosine hydroxylase, the product tyrosine contains tritium in the 3-position of the aromatic ring (Daly et al., 1968), due to an NIH shift of the isotope. An NIH shift also occurs with several of the substituents characterized in this study. Oxygen addition at the 4-position of the substrate results in formation of tyrosine or 3-X-tyrosines due to elimination of the substituent or an NIH shift, respectively. Halogen elimination to form tyrosine by tyrosine hydroxylase occurs with fluorine, chlorine, and bromine, in the order F $\gg$ Cl > Br. An NIH shift can occur with Cl, Br, CH_3, and H. These migratory aptitudes are most consistent with a cationic species. Thus, the degree of migration by the halogens decreases with increasing electronegativity and decreasing ability to form a three-centered transition state. The ready ability of H and CH_3 to migrate

[3] An alternate possibility to immediate formation of a CO bond would be abstraction of a single electron from the aromatic ring to form a π-cation followed by rebound of the activated oxygen onto the aromatic ring. However, such a mechanism would not be expected to generate deuterium kinetic isotope effects greater than 1. Deuterium isotope effects of 1.5−1.8 have been reported for hydroxylation of deuterated phenylalanines by both tyrosine (Fitzpatrick, 1994) and phenylalanine hydroxylase (Abita et al., 1984). Such results can be explained if the formation of the CO bond shown in Scheme 3 is coupled to weakening of the CH(D) bond.

Scheme 5



is also much more consistent with a cationic intermediate than with a neutral radical. Direct elimination to form tyrosine occurs as the alternative to the NIH shift and must be less sensitive to the electronegativity of the substituent.

A study by Kurata et al. (1988) of the NIH shift during one-electron oxidation of substituted benzenes provides support for the mechanism proposed in Scheme 3. They interpreted their results in support of the NIH shift occurring from a cation as shown in Scheme 5. Such a mechanism would be fully consistent with the results presented here.

The mechanism in Scheme 3 is written with a proposed iron−oxo species, although no evidence is presented here for the existence of such an intermediate. A 4a-peroxytetrahydropterin has previously been invoked as the product of the reaction of oxygen and tetrahydropterin in the active site of tyrosine hydroxylase (Kaufman & Kaufman, 1985). One can propose that the 4a-peroxytetrahydropterin is the hydroxylating intermediate in tyrosine hydroxylase, in analogy to the 4a-peroxyflavin known to be an intermediate with the flavoprotein phenol hydroxylases (Ballou, 1982). A critical difference between the flavin- and pterin-dependent hydroxylases is the presence of iron in the latter. The requirement of tyrosine hydroxylase for the iron for catalytic activity is demonstrated by the lack of activity of apoenzyme (Fitzpatrick, 1989; Haavik et al., 1991) or of mutant proteins in which ligands to the iron have been mutated (Ramsey et al., 1995). However, the role of the iron remains obscure. The similarity in the degree of coupling with two different tetrahydropterins indicates hydroxylation is relatively insensitive to the tetrahydropterin used. An alternative to a peroxytetrahydropterin is an iron−oxo species. As shown here, tyrosine hydroxylase can catalyze benzylic hydroxylation, forming 4-(hydroxymethyl)phenylalanine from 4-methylphenylalanine (Table 2). This type of nonaromatic hydroxylation is more consistent with known reactions carried out by iron-containing enzymes, such as the P450-containing monooxygenases, than with peroxide chemistry and lends further support to an iron-containing hydroxylating species.

In conclusion, the results presented here provide several new insights into the mechanism of tyrosine hydroxylase. The transition state for hydroxylation is highly electron deficient, consistent with development of a cationic intermediate. The hydroxylating species discriminates between C-3 hydroxylation and C-4 hydroxylation on the basis of the size of the substituent at the C-4 position. Lastly, the NIH shift occurs with functional groups in a pattern consistent with a cationic species. The simplest mechanism

consistent with the data is formation of a highly reactive, electron deficient oxygen species reacting with the least hindered site on the aromatic ring, followed by rearrangement and rearomatization of the ring to form the hydroxylated product.

SUPPORTING INFORMATION AVAILABLE

Syntheses and characterization of the amino acids used in this study (7 pages). Ordering information is given on any current masthead page.

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