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Steady-State Kinetic Mechanism of Rat Tyrosine Hydroxylase†

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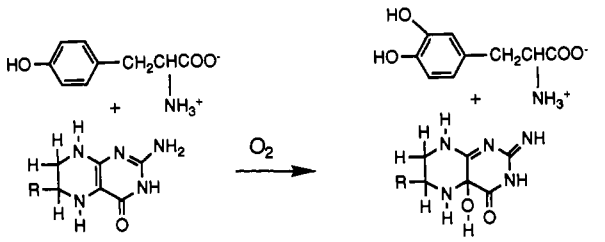
Received September 13, 1990; Revised Manuscript Received November 28, 1990

ABSTRACT: The steady-state kinetic mechanism for rat tyrosine hydroxylase has been determined by using recombinant enzyme expressed in insect tissue culture cells. Variation of any two of the three substrates, tyrosine, 6-methyltetrahydropterin, and oxygen, together at nonsaturating concentrations of the third gives a pattern of intersecting lines in a double-reciprocal plot. Varying tyrosine and oxygen together results in a rapid equilibrium pattern, while the other substrate pairs both fit a sequential mechanism. When tyrosine and 6-methyltetrahydropterin are varied at a fixed ratio at different oxygen concentrations, the intercept replot is linear and the slope replot is nonlinear with a zero intercept, consistent with rapid equilibrium binding of oxygen. All the replots when oxygen is varied in a fixed ratio with either tyrosine or 6-methyltetrahydropterin are nonlinear with finite intercepts. 6-Methyl-7,8-dihydropterin and norepinephrine are competitive inhibitors versus 6-methyltetrahydropterin and noncompetitive inhibitors versus tyrosine. 3-Iodotyrosine, a competitive inhibitor versus tyrosine, shows uncompetitive inhibition versus 6-methyltetrahydropterin. At high concentrations, tyrosine is a competitive inhibitor versus 6-methyltetrahydropterin. These results are consistent with an ordered kinetic mechanism with the order of binding being 6-methyltetrahydropterin, oxygen, and tyrosine and with formation of a dead-end enzyme–tyrosine complex. There is no significant primary kinetic isotope effect on the V/K values or on the V_{max} value with [3,5-$^2H_2$]tyrosine as substrate. No burst of dihydroxyphenylalanine production is seen during the first turnover. These results rule out product release and carbon–hydrogen bond cleavage as rate-limiting steps.

Tyrosine hydroxylase catalyzes the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA)[1] (Scheme I), the first step in the biosynthesis of catecholamine neurotransmitters (Kaufman & Kaufman, 1985). Besides tyrosine, the physiological substrates for the enzyme are molecular oxygen and tetrahydrobiopterin; a 4a-hydroxytetrahydropterin is the other product (Dix et al., 1987; Haavik & Flatmark, 1987). Tyrosine hydroxylase is known to be an iron protein; the active form contains one ferrous iron atom per subunit (Dix et al., 1985; Fitzpatrick, 1989). The role of the iron is unknown, but attempts to replace it with other metals have not been successful (Fitzpatrick, 1989).

The kinetic mechanism of bovine tyrosine hydroxylase has been studied by several workers, with contradictory results. Thus, Ikeda et al. (1966), using a protease-treated enzyme, reported that varying 6,7-dimethyltetrahydropterin and tyrosine together resulted in a series of parallel lines in a double-reciprocal plot. They concluded that the enzyme used a ping-pong mechanism involving a reduced enzyme intermediate. However, Kaufman and Fisher (1974) reported unpublished experiments in which an intersecting line pattern was obtained with the same substrates and concluded that the mechanism involved a quaternary complex of enzyme with all three substrates. Oka et al. (1981) reported that varying tetrahydrobiopterin and oxygen or tetrahydrobiopterin and tyrosine together gave intersecting line patterns, but varying

Scheme I



tyrosine and oxygen together gave parallel lines. In all cases, the double-reciprocal plots were concave down. In contrast, Bullard and Capson (1983) found intersecting line patterns with all three substrate pairs, with no sign of curvature. On the basis of product inhibition studies, these authors concluded that either tyrosine or tetrahydrobiopterin could bind to the enzyme first but that oxygen must bind after tetrahydrobiopterin. Recently, we have reported that tyrosine must be able to bind after the tetrahydropterin and that both DOPA and 5-deazatetrahydropterin, two of the inhibitors used by Bullard and Capson (1983), can bind to multiple forms of the bovine enzyme (Fitzpatrick, 1988).

The difficulty of obtaining significant amounts of purified tyrosine hydroxylase has undoubtedly contributed to the contradictory results reported to date. We have recently ex-

†This research was supported in part by National Science Foundation Grant DMB-8816407.

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; DOPA, dihydroxyphenylalanine; 6-MePH$_4$, 6-methyltetrahydropterin; 6-MePH$_2$, 6-methyl-7,8-dihydropterin.

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Table I: Kinetic Parameters for Rat Tyrosine Hydroxylase[a]

varied substrates	fixed substrate	pattern	apparent kinetic parameters			
			V_{max} (min^{-1})	K_{MPH_4} (μM)	K_{O_2} (μM)	K_{Tyr} (μM)
tyrosine/6-$MePH_4$	oxygen (250 μM)	sequential	92 ± 11	46 ± 13		32 ± 10
6-$MePH_4$/O_2	tyrosine (150 μM)	sequential	75 ± 8	30 ± 8	21 ± 19	
tyrosine/O_2	6-$MePH_4$ (500 μM)	equilibrium ordered	91 ± 4.9			22 ± 4

[a] Conditions: 14 mM β-mercaptoethanol, 75 μg/mL catalase, 10 μM ferrous ammonium sulfate, 50 mM sodium acetate, 50 mM MES, and 100 mM Tris-HCl, pH 6.5, at 30 °C.

pressed rat tyrosine hydroxylase at high levels in insect cells using a baculovirus expression system (Fitzpatrick et al., 1990), affording ample amounts of material for mechanistic studies. Using the recombinant enzyme, we have determined the steady-state kinetic mechanism, a necessary first step in a mechanistic study. The results are reported here.

Experimental Procedures

[3,5-^{3}H]Tyrosine was from either New England Nuclear or Amersham Corp.; it was purified before use by the method of Ikeda et al. (1966). [3,5-$^2H_2$]Tyrosine was from Merck; the concentrations of stock solutions for kinetic isotope effect measurements were determined spectrophotometrically by using a value of ϵ_{274} = 1.39 mM^{-1} cm^{-1} (Fasman, 1989). Catalase was from Boehringer-Mannheim. 6-Methyltetrahydropterin was synthesized as described previously (Fitzpatrick, 1988); the concentration was determined from the absorbance at 266 nm in 2 M perchloric acid by using an extinction coefficient of 17.8 mM^{-1} cm^{-1} (Shiman et al., 1971). Stock solutions were made up fresh daily in 5 mM HCl. 6-Methyl-7,8-dihydropterin was synthesized by the method of Mager et al. (1967); the concentration of stock solutions was determined in 2 M perchloric acid by using a value of ϵ_{356} = 4060 M^{-1} cm^{-1}. Solutions of ferrous ammonium sulfate were made up as 1 mM stocks at pH 3 and stored at −20 °C until thawed for use.

The assay of tyrosine hydroxylase was based on the release of tritium from [3,5-^{3}H]tyrosine as previously described (Fitzpatrick, 1989). Standard assay conditions were 75 μg/mL catalase, 14 mM β-mercaptoethanol, 10 μM ferrous ammonium sulfate, 50 mM sodium acetate, 50 mM MES, and 100 mM Tris-HCl, at pH 6.5, 30 °C, in a volume of 1 mL. Assays were started by adding either 5-methyltetrahydropterin or enzyme. After 2 min at 30 °C, the reaction was quenched by adding 0.05 mL of acetic acid, and the tritiated water was separated on a Dowex column. When the concentration of oxygen was to be varied, the assay mix was stirred in a sealed, thermostated chamber under a stream of the desired concentration of oxygen mixed with nitrogen. Ferrous ammonium sulfate was added to the assay mix immediately before attaching the septum. Assays were started by injecting tetrahydropterin through the septum, followed within 30 s by enzyme. For assays at 1.18 mM oxygen, the concentration of catalase was increased to 150 μg/mL, and the assay time was decreased to 30 s. Burst kinetic measurements were done at 2 °C at pH 7 with 150 μM tyrosine, 500 μM 6-methyltetrahydropterin, and 440 μM oxygen. Assays were started by adding 6-methyltetrahydropterin or enzyme to the other assay components stirring in a conical tube. The order of addition had no effect on the results. The reactions were quenched at the indicated times by adding 0.1 mL of 50% acetic acid and analyzed as described above.

To determine kinetic isotope effects, a modification of the procedure of Arnow (1937) was used to determine the amount of DOPA formed. Incubation conditions were as described above for the standard assay, except that the assay volume was reduced to 0.5 mL and the reaction was quenched with 0.015 mL of 50% HCl. To this was added 0.2 mL of 12.5% sodium nitrite and 12.5% sodium molybdate. After 10–15 min at room temperature, 0.07 mL of 3 M NaOH was added, and the absorbance at 500 nm was determined 10 s later. Because of the instability of the chromophore, it was necessary to consistently determine the absorbance at the same time after adding base for each sample. For each set of assays a standard curve from 0.75 to 6.25 nmol of DOPA was run. Controls established that neither tyrosine nor 6-methyltetrahydropterin interfered with this assay.

Rat tyrosine hydroxylase expressed in insect tissue culture cells was purified as described previously (Fitzpatrick et al., 1990). Protein concentrations were determined by using a value of $\epsilon_{280}^{1\%}$ of 10.4 (Haavik et al., 1988) and a subunit molecular weight of 56 000. Steady-state kinetic data were fit to the relevant programs of Cleland (1979) by using programs adapted for use on a Macintosh microcomputer by Dr. James Robertson of The Pennsylvania State University.

Results

Steady-State Kinetics. The three substrates for tyrosine hydroxylase are tyrosine, oxygen, and a tetrahydropterin. 6-Methyltetrahydropterin was chosen as the tetrahydropterin for examination of the steady-state kinetics because the K_M values for the substrates are in a more accessible range than is the case when the physiological substrate (6*R*)-tetrahydrobiopterin is used (Fitzpatrick et al., 1990). Each pair of substrates was varied at a nonsaturating concentration of the third substrate at pH 6.5. Variation of tyrosine and 6-methyltetrahydropterin together at 250 μM oxygen gave a pattern of intersecting lines in a double-reciprocal plot; the data were best fit with a sequential mechanism. Similar results were obtained when oxygen and 6-methyltetrahydropterin were varied together at 150 μM tyrosine. In contrast, the data when tyrosine and oxygen were varied together at 500 μM 6-methyltetrahydropterin were best fit with an equilibrium ordered mechanism. The results of these experiments are summarized in Table I. These results establish that the mechanism involves a quaternary complex of all three substrates with the enzyme. In no case was substrate inhibition seen with oxygen as substrate, in contrast to the report of Oka et al. (1981). However, if assays at 1.2 mM oxygen were carried out for 2 min, apparent oxygen inhibition was seen due to the nonlinearity of DOPA production over this time period (results not shown).

The complete rate equation for a three-substrate reaction involving a ternary complex can contain eight terms (eq 1). While the individual terms can be evaluated by varying all three substrates at once, such an approach demands extremely high precision. Alternatively, one can saturate in each substrate and vary the other two. However, tyrosine exhibits substrate inhibition at concentrations above 150 μM, while the decreased precision due to shortened assay times at concentrations of oxygen above ambient combined with the low apparent K_M value for oxygen ruled out such an approach with

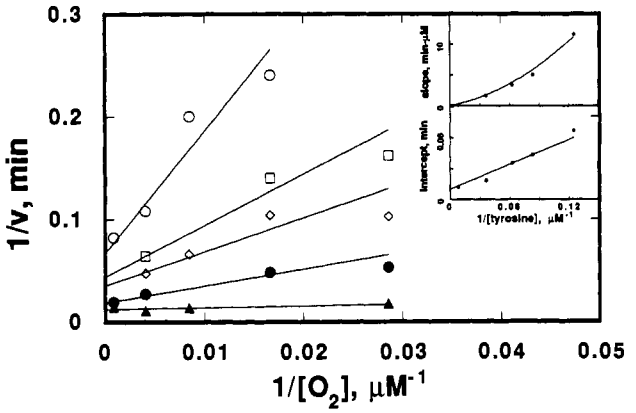


FIGURE 1: Initial velocity patterns vs oxygen concentration at a fixed ratio of tyrosine and 6-methyltetrahydropterin concentrations. Conditions are as in Table I with (▲) 207 μM 6-methyltetrahydropterin and 135 μM tyrosine, (○) 48.3 μM 6-methyltetrahydropterin and 31.5 μM tyrosine, (●) 27.6 μM 6-methyltetrahydropterin and 18 μM tyrosine, and (△) 17.3 μM 6-methyltetrahydropterin and 11.3 μM tyrosine. The insets are plots of the relevant slopes or intercepts vs the reciprocal tyrosine concentration.

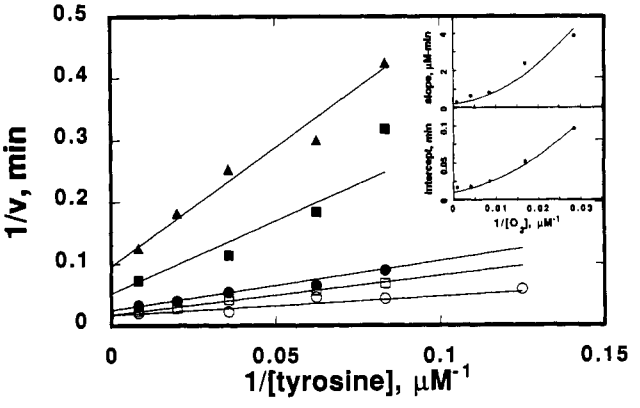


FIGURE 2: Initial velocity patterns vs tyrosine concentration at a fixed ratio of oxygen and 6-methyltetrahydropterin concentrations. Conditions are as in Table I with (○) 374 μM 6-methyltetrahydropterin and 1180 μM oxygen, (□) 78.5 μM 6-methyltetrahydropterin and 246 μM oxygen, (●) 37.4 μM 6-methyltetrahydropterin and 118 μM oxygen, (■) 18.7 μM 6-methyltetrahydropterin and 60 μM oxygen, and (▲) 9.4 μM 6-methyltetrahydropterin and 32 μM oxygen. The insets are plots of the relevant slopes or intercepts vs the reciprocal oxygen concentration.

that substrate. Instead, the method of Rudolph and Fromm (1979) was used, in which two substrates are varied in a fixed ratio at different concentrations of the third. The presence of individual terms in the rate equation can be determined from the shapes of the slope and intercept replots by using such an analysis, since terms containing the concentrations of the two substrates in fixed ratio will contain squared terms, generating nonlinear replots.

$$v = V[\mathrm{Tyr}][\mathrm{O_2}][6\mathrm{MPH_4}]/([\mathrm{Tyr}][\mathrm{O_2}][6\mathrm{MPH_4}] + K_{\mathrm{Tyr}}[\mathrm{O_2}][6\mathrm{MPH_4}] + K_{\mathrm{O_2}}[\mathrm{Tyr}][6\mathrm{MPH_4}] + K_{\mathrm{MPH_4}}[\mathrm{Tyr}][\mathrm{O_2}] + \mathrm{coef(Tyr)}[\mathrm{Tyr}] + \mathrm{coef}(6\mathrm{MPH_4})[6\mathrm{MPH_4}] + \mathrm{coef}(\mathrm{O_2})[\mathrm{O_2}] + \mathrm{constant}) \quad (1)$$

In all cases, variation of two of the substrates in a fixed ratio gave a pattern of intersecting lines in a double-reciprocal plot (Figures 1–3), consistent with a quaternary complex mechanism. When tyrosine and 6-methyltetrahydropterin were varied in a fixed ratio, the slope replot was nonlinear and passed through the origin (Figure 1). The intercept replot was linear, with a finite intercept. The y-intercept of the slope replot equals $K_{\mathrm{O_2}}/V$; a value of zero is predicted for this from the equilibrium ordered pattern seen when tyrosine and oxygen were varied together (Table I). The nonlinearity of the slope replot is consistent with a finite value for the constant term in eq 1. A linear intercept replot indicates that the coef(O_2) term in eq 1 is zero. When oxygen and tyrosine or oxygen and 6-methyltetrahydropterin were varied in a fixed ratio, both the slope and intercept replots were nonlinear (Figures 2 and 3). The finite intercepts for the slope replots establish that the values of K_{Tyr}/V and $K_{\mathrm{MPH_4}}/V$ are finite. The nonlinear intercept replots are consistent with nonzero values for the coef(Tyr) and coef($6MPH_4$) terms, while the nonlinear slope replots again indicate that the constant term is not zero. These results establish that the steady-state kinetic equation for rat tyrosine hydroxylase is given by eq 2.

$$v = V[\mathrm{Tyr}][\mathrm{O_2}][6\mathrm{MPH_4}]/([\mathrm{Tyr}][\mathrm{O_2}][6\mathrm{MPH_4}] + K_{\mathrm{Tyr}}[\mathrm{O_2}][6\mathrm{MPH_4}] + K_{\mathrm{MPH_4}}[\mathrm{Tyr}][\mathrm{O_2}] + \mathrm{coef(Tyr)}[\mathrm{Tyr}] + \mathrm{coef}(6\mathrm{MPH_4})[6\mathrm{MPH_4}] + \mathrm{constant}) \quad (2)$$

The turnover number and Michaelis constants can be determined by using this equation with the data for Table I. Due to the lack of a $K_{\mathrm{O_2}}$ term, the apparent value of V_{max} determined by varying both tyrosine and 6-methyltetrahydropterin at a fixed oxygen concentration equals the actual value, 92

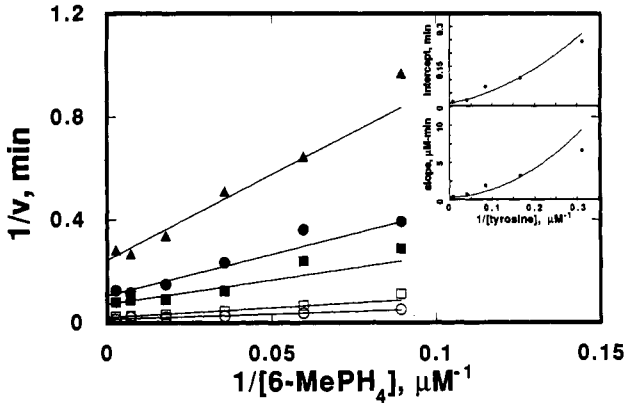


FIGURE 3: Initial velocity patterns vs 6-methyltetrahydropterin concentration at a fixed ratio of tyrosine and oxygen concentrations. Conditions are as in Table I with (○) 118 μM tyrosine and 1180 μM oxygen, (□) 24.6 μM tyrosine and 246 μM oxygen, (■) 11.8 μM tyrosine and 118 μM oxygen, (●) 6 μM tyrosine and 60 μM oxygen, and (▲) 3.2 μM tyrosine and 32 μM oxygen. The insets are plots of the relevant slopes or intercepts vs the reciprocal tyrosine concentration.

min^{-1}. Similarly, the value of $K_{\mathrm{MPH_4}}$ determined by varying oxygen and 6-methyltetrahydropterin together, 30 μM, is the intrinsic value, and the value of K_{Tyr} obtained by varying tyrosine and oxygen together, 22 μM, is the intrinsic value. These values were used to determine the remaining kinetic constants from the results by using eq 2; coef(MPH_4) = 1730 μM^2, coef(Tyr) = 1710 μM^2, and the constant term is 8.7×10^4 μM^3.

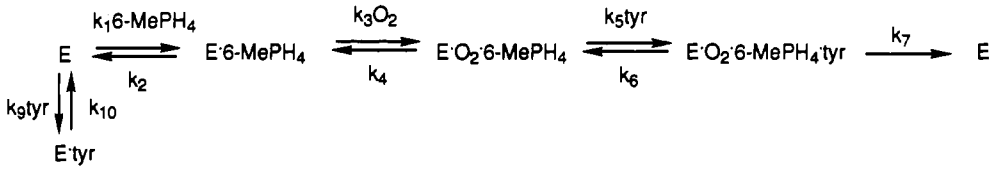
Inhibition Studies. Equation 2 is consistent with several possible ordered kinetic mechanisms for tyrosine hydroxylase. Inhibition studies with dead-end and product inhibitors were used to determine whether tyrosine or 6-methyltetrahydropterin bound first to the enzyme. The results are summarized in Table II. 6-Methyl-7,8-dihydropterin is a competitive inhibitor versus 6-methyltetrahydropterin and a noncompetitive inhibitor versus tyrosine. Norepinephrine shows a similar pattern, consistent with a number of reports that catechols are competitive inhibitors versus tetrahydropterins with this enzyme (Kaufman & Kaufman, 1985; Fitzpatrick, 1988). 3-I-tyrosine is a competitive inhibitor versus tyrosine and an uncompetitive inhibitor versus 6-methyltetrahydropterin. The product DOPA is a noncompetitive inhibitor versus both

Table II: Inhibition Patterns with Dead-End Inhibitors for Rat Tyrosine Hydroxylase[a]

varied substrate	inhibitor	fixed substrate	inhibition pattern	K_{is} (μM)	K_{ii} (μM)
6-MePH$_4$	6-MePH$_2$	20 μM tyrosine	C	53 ± 5	
6-MePH$_4$	6-MePH$_2$	150 μM tyrosine	C	42 ± 5	
tyrosine	6-MePH$_2$	48 μM 6-MePH$_4$	NC	340 ± 170	230 ± 46
tyrosine	6-MePH$_2$	203 μM 6-MePH$_4$	NC	880 ± 520	340 ± 71
6-MePH$_4$	norepinephrine	40 μM tyrosine	C	4.3 ± 1.2	
tyrosine	norepinephrine	34 μM 6-MePH$_4$	NC	50 ± 28	12 ± 2.6
6-MePH$_4$	I-tyrosine	20 μM tyrosine	UC		0.15 ± 0.004
6-MePH$_4$	I-tyrosine	150 μM tyrosine	UC		1.6 ± 0.47
tyrosine	I-tyrosine	50 μM 6-MePH$_4$	C	0.069 ± 0.028	
tyrosine	I-tyrosine	500 μM 6-MePH$_4$	C	0.099 ± 0.011	
tyrosine	DOPA	32 μM 6-MePH$_4$	NC	130 ± 54	35 ± 3.7
6-MePH$_4$	DOPA	25 μM tyrosine	NC	140 ± 65	170 ± 38

[a] Conditions: 14 mM β-mercaptoethanol, 75 μg/mL catalase, 10 μM ferrous ammonium sulfate, 50 mM MES, 50 mM sodium acetate, 0.25 mM oxygen, and 100 mM Tris-HCl, pH 6.5, at 30 °C.

Scheme II



tyrosine and 6-methyltetrahydropterin. The other product, the 4a-hydroxytetrahydropterin, is rapidly converted to the dihydropterin in solution, so it could not be tested directly as an inhibitor. All of the inhibition results are consistent with ordered addition of 6-methyltetrahydropterin before tyrosine. The noncompetitive inhibition patterns seen with DOPA are consistent with DOPA being the first product released.

Tyrosine shows substrate inhibition at high levels. To determine the type of inhibition, initial rates were measured at different concentrations of 6-methyltetrahydropterin at concentrations of tyrosine sufficiently high that the only effect was inhibitory. Under these conditions, tyrosine is a competitive inhibitor versus 6-methyltetrahydropterin (results not shown).

Kinetic Isotope Effects. During the reaction catalyzed by tyrosine hydroxylase, the carbon–hydrogen bond at the 3-position of the aromatic ring of tyrosine is cleaved. To determine if this occurred in a slow step, kinetic isotope effects upon the steady-state rate constants were determined with [3,5-$^2H_2$]tyrosine as substrate. As summarized in Table III, no isotope effects significantly different from one were found.[2]

Burst Kinetics. To determine if the rate-limiting step occurred after oxygen transfer to tyrosine, the rate of DOPA formation during the first few enzyme turnovers was determined. This was done at 2 °C to slow the reaction down sufficiently to be able to detect any burst. At saturating levels of tyrosine and oxygen and high levels of tyrosine, there was no detectable burst of DOPA formation (results not shown).

Discussion

The data presented here are consistent with the mechanism of Scheme II for rat tyrosine hydroxylase. The results of varying the substrates pairwise at nonsaturating concentrations of the third substate establish that the mechanism involves a quaternary complex of enzyme and all three substrates. Varying substrates pairwise in fixed ratios establishes that the coef(O_2) term is not significant. Both approaches are consistent with oxygen adding in rapid equilibrium fashion. These results establish eq 2 as the steady-state rate equation for tyrosine hydroxylase. This equation is consistent with an ordered mechanism in which either tyrosine or 6-methyltetrahydropterin binds first to the enzyme and oxygen binds second. The inhibition patterns given in Table II clearly establish that 6-methyltetrahydropterin binds before tyrosine. This is the same order of binding for these two substrates that has been established for the bovine enzyme, although the complete kinetic mechanism has not been determined in that case (Fitzpatrick, 1988).

Table III: Kinetic Isotope Effects on Rat Tyrosine Hydroxylase at pH 6.5[a]

parameter	isotope effect
V	0.93 ± 0.06[b,c]
	1.04 ± 0.04[b,d]
	0.96 ± 0.09[b,e]
	0.98 ± 0.06 (av)
V/K_{MPH_4}	1.00 ± 0.10[c,f]
V/K_{tyr}	1.02 ± 0.06[d,f]

[a] Isotope effects were determined by direct comparison of the rates obtained with tyrosine and [3,5-$^2H_2$]tyrosine under the conditions of Tables I and II. [b] Calculated assuming no isotope effect on V/K. [c] Varied 6-MePH$_4$, with 150 μM tyrosine and 250 μM O_2. [d] Varied tyrosine, with 840 μM 6-MePH$_4$ and 250 μM O_2. [e] Varied O_2, with 560 μM 6-MePH$_4$ and 150 μM tyrosine. [f] Calculated assuming no isotope effect on V_{max}.

The rapid equilibrium addition of oxygen would normally result in the coef(Tyr) term also being missing from the rate equation, due to $k_3 \gg k_2$. However, dead-end inhibition due to binding of the tyrosine to the free enzyme results in the presence of this term in the rate equation. The ability of such a dead-end complex to form is demonstrated directly by the competitive inhibition versus 6-methyltetrahydropterin seen at very high tyrosine concentrations.

Since both oxygen and 6-methyltetrahydropterin bind before tyrosine, one must consider the possibility that a hydroxylating intermediate could form in the absence of an amino acid substrate. Indeed, Dix et al. (1987) have reported that tetrahydrobiopterin and oxygen can react with tyrosine hydroxylase in the absence of tyrosine to form a species competent for hydroxylation and that tyrosine hydroxylase cata-

[2] It should be noted that the commonly used assay for tyrosine hydroxylase measures the rate of release of label from [3,5-^{3}H]tyrosine, so that any significant kinetic isotope effect on the V/K_{Tyr} value would invalidate this assay. We have measured the $^TV/K$ value of tyrosine with bovine adrenal tyrosine hydroxylase and found a value of 1.03 ± 0.06 in that case (Meyer, 1989).

lyzes tetrahydrobiopterin oxidation in the absence of tyrosine at pH 8.2, but much less at pH 7.2. At pH 6.5, we have been unable to detect any increase in 6-methyltetrahydropterin or tetrahydrobiopterin oxidation over background in the absence of an amino acid substrate.[3] However, the results at the higher pH values are consistent with the kinetic mechanism proposed here.

Kinetic isotope effects were measured with [3,5-$^2H_2$]tyrosine as substrate to determine if carbon–hydrogen bond cleavage occurred in a partially rate-limiting step. Since no significant isotope effect was seen on the V/K_{Tyr} value, carbon–hydrogen bond cleavage either occurs after the first irreversible step or is much faster than another step preceding or including the first irreversible step. The lack of an observed isotope effect on the V_{max} value indicates that carbon–hydrogen bond cleavage is much faster than either product release or a chemical step. This result is in contrast to the kinetic isotope effect of 1.5 seen with phenylalanine hydroxylase when [$^2H_5$]phenylalanine is used as a substrate (Abita et al., 1984). To determine directly if product release is rate limiting, the rate of production of DOPA during the first few enzyme turnovers was determined. No burst of DOPA formation was seen, establishing that the rate-limiting step occurs before or at the time of oxygen transfer to tyrosine. Therefore, the lack of a kinetic isotope effect on the V_{max} value must be due to carbon–hydrogen bond cleavage being much faster than either another chemical step or a conformational change.

Such a result is consistent with a number of hydroxylation mechanisms. Electrophilic attack on the aromatic ring of tyrosine by an activated oxygen intermediate is a reasonable first step in hydroxylation; such a step is expected to be irreversible, so that no V/K isotope effect will be seen. Carbon–hydrogen bond cleavage would occur during the subsequent tautomerization of a hydroxycyclohexadienone-like intermediate; such a step is expected to be quite rapid, so that no V_{max} isotope effect would be expected. The initial attack upon the aromatic ring might show an inverse secondary isotope effect. The data of Table III are not precise enough to rule out a secondary effect of less than 5%. The data do rule out a mechanism involving direct hydrogen atom abstraction before or concerted with oxygen addition.

In summary, the results described here provide a number of insights into the mechanism of tyrosine hydroxylase: (1) The steady-state kinetic mechanism has been determined. 6-Methyltetrahydropterin, oxygen, and tyrosine bind in an ordered fashion, forming a quaternary complex before any irreversible step occurs. In addition, tyrosine can bind to the free enzyme to form a dead-end complex. (2) The rate-limiting step is not product release or carbon–hydrogen bond cleavage. The simplest mechanism consistent with the data is that the rate-limiting step occurs before cleavage of the tyrosine meta carbon–hydrogen bond.

[3] P. F. Fitzpatrick, unpublished observations.

ACKNOWLEDGMENTS

I thank Dr. Paul Cook for helpful comments on the manuscript.

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