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Steady-State Kinetics of Bovine Striatal Tyrosine Hydroxylase

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SUMMARY

The steady-state kinetics of tyrosine hydroxylase [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] frequently exhibits complex features which confound interpretation of the results. Using an assay-enzyme system which is essentially devoid of the major mitigating kinetic features, a comprehensive kinetics data base has been compiled. The studies employed L-tyrosine, 5,6,7,8-tetrahydrobiopterin, and oxygen as substrates, and 3-(3′,4′-dihydroxyphenyl)L-alanine, a deazapterin, 3-iodo-L-tyrosine, and dopamine as product, substrate analogue, and product analogue inhibitors, respectively. All three reactants were varied pairwise, and all inhibitors (except dopamine) were tested with each of the three substrates as variable substrate. The entire data base was interpreted exclusively in terms of models for classic saturation kinetics of enzyme catalysis, providing an internally consistent kinetic model and evidence for a sequential mechanism with partially ordered sequences for substrate addition and product release. Some possible mechanisms and experimental variables relating these results to more complex kinetics of tyrosine hydroxylase are considered briefly.

INTRODUCTION

The enzymatic conversion of TYR[1] to L-dopa is catalyzed by tyrosine hydroxylase (1), and is the rate-controlling step in the biosynthesis of catecholamine neurotransmitters (2) of the central and peripheral nervous systems. Regulation of tyrosine hydroxylase activity has been studied extensively, but the catalytic mechanism remains largely uncharacterized. Studies on the steady-state kinetics of tyrosine hydroxylase catalysis have been limited to examination of only a few variables (3–6) and often have been performed using the synthetic cofactors 6,7-dimethyl-5,6,7,8-tetrahydropterin and 6-MPH$_4$, which have been shown to behave differently from the natural cofactor, BH$_4$, in this hydroxylating system (7).

The kinetics of tyrosine hydroxylase catalysis is often complicated by nonlinear double-reciprocal relationships which in some cases suggest multiple forms of the enzyme or cooperativity (8, 9), and in others more clearly reflect substrate inhibition which has been observed at high levels of the pteridine cofactor, tyrosine or oxygen (10–12). The kinetic behavior of tyrosine hydroxylase can also vary with assay conditions, and is influenced significantly by the stereochemical configurations around carbon 6 and within the C-6 substituent of the pteridine cofactors (12, 13).

The partially purified bovine striatal enzyme and assay conditions used in these studies constitute a system which is essentially devoid of the complex nonlinear functions and thus lends itself to classical steady-state kinetic analysis. Using catalytically reduced biopterin, which is a mixture of the 6 S and 6 R diastereomers (12) of the natural cofactor, BH$_4$, the steady-state kinetics of partially purified bovine striatal tyrosine hydroxylase have been examined. Velocity measurements with variations in TYR, BH$_4$, and O$_2$ concentrations, along with product and dead-end inhibition patterns against each substrate, provide information on the order of substrate addition and product release steps in the catalytic sequence.

MATERIALS AND METHODS

Enrichment of bovine striatal tyrosine hydroxylase. Bovine brains were obtained at a local slaughterhouse. Striata were rapidly dissected on location, frozen on dry ice, and stored at −70° until used. Striatal tyrosine hydroxylase was partially purified by chromatography on heparin/agarose (Bethesda Research Laboratories, Bethesda, Md.) and concentrated by ammonium sulfate precipitation. The procedures were executed at 0–4° and required 9 hr. One hundred grams of striatal tissue were homogenized in three volumes of 5 mM potassium phosphate buffer (pH 7) (hereafter referred to as phosphate buffer) for 90 sec in a food processor. The homogenate was centrifuged at 28,000 × *g* for 30 min, and the supernatant (290 ml) was applied to a bed (3.8 × 10 cm) of

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[1] The abbreviations used are: TYR, L-tyrosine; L-dopa, 3-(3′,4′-dihydroxyphenyl-L-alanine; 6-MPH$_4$, 6-methyl-5,6,7,8-tetrahydropterin; BH$_4$, 5,6,7,8-tetrahydrobiopterin; 3-IT, 3-iodo-L-tyrosine; DAP, 5-deaza-5,6,7,8-tetrahydro-6-methylpterin; BH$_2$, dihydropterin (exact structure not established).

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heparin/agarose equilibrated with phosphate buffer. The column was washed with two bed-volumes of phosphate buffer and then with phosphate buffer containing 200 mM NaCl until the optical density of the eluate at 280 nm was zero. Tyrosine hydroxylase was eluted with two bed-volumes each of phosphate buffer containing 400 mM NaCl and 600 mM NaCl, the major peak of activity occurring in the second bed-volume of 400 mM NaCl. Over-all yield varied from 75% to 95%, and an average increase in specific activity was 10- to 15-fold. Tyrosine hydroxylase eluted from the heparin/agarose column was concentrated by precipitation with 40% ammonium sulfate and resuspension to 0.05 time the original volume in phosphate buffer. The resuspended enzyme was frozen in liquid nitrogen and stored at −70°. Ammonium sulfate precipitation resulted in a nearly quantitative recovery of tyrosine hydroxylase with a 0.5- to 2-fold increase in specific activity. Final specific activity ranged from 20 to 30 nmoles of L-dopa formed per milligram per minute under conditions of saturating L-tyrosine and BH_4 and ambient O_2 tension. Kinetic studies were performed with the concentrated tyrosine hydroxylase preparation. Protein measurement for specific activity was carried out by the method of Lowry *et al.* (14), using crystalline bovine serum albumin (Sigma Chemical Company, St. Louis, Mo.) as standard.

Tyrosine hydroxylase assays. Tyrosine hydroxylase activity was measured by the tritium-release method of Nagatsu *et al.* (15) in a 0.35-ml reaction mixture containing 143 mM sodium succinate buffer (pH 5.9), 2000 units of bovine liver catalase, 7 mM dithiothreitol, 1 μCi of 3,5-ditritio-L-tyrosine, L-tyrosine and BH_4 as indicated, and 15–60 μg of the tyrosine hydroxylase preparation described above. Incubations were carried out for 6 min in a water bath at 37°. The reaction was stopped by cooling to 0° and adding 100 μl of glacial acetic acid. Tritiated water formed in the reaction was isolated by passing 350 μl of the quenched reaction mixture over a Dowex 50-H^+ column (0.5 × 4 cm) and quantitative elution with two 0.6-ml volumes of distilled water. The total eluate was collected in a scintillation vial. After the addition of 13 ml of scintillation cocktail (ACS, Amersham Corporation, Arlington Heights, Ill.), tritium was measured by scintillation counting in a Packard Model 3385 liquid scintillation spectrometer. Reaction blanks routinely contained a complete reaction mixture and 2 mM 3-IT, a potent tyrosine hydroxylase inhibitor (16). Blanks composed of reaction mixtures in which tyrosine hydroxylase or BH_4 was excluded were identical with each other and with the blanks containing 3-IT. Blank values were typically 600–1200 cpm, and net activity ranged from 5 to 30 times that of the blank depending on reaction conditions. The assay was linear with both time and protein concentration in the ranges used. All assays and blanks were performed in duplicate except as noted for Fig. 1. The amount of enzyme used in a set of assays was selected to give maximal net activity and at the same time to permit no more than 10% conversion of the limiting substrate to product during the incubation. With the exception of assays involving the lowest O_2 and highest TYR concentrations, the concentration of TYR was always limiting.

Sheep liver quinonoid-dihydropteridine reductase/NADH, β-mercaptoethanol, and dithiothreitol were compared as reductants in the assay. The tyrosine hydroxylase reaction velocity and linearity with time were not significantly different for the three reducing systems. Since neither chemical nor enzymatic (17) regeneration of BH_4 will alter the ratio of 6 R and 6 S isomers during the reaction, and since the quinonoid-dihydropteridine reductase/NADH regenerating system offered no apparent advantage over the sulfhydryl reagents, dithiothreitol was selected. It is in principle a more effective reductant and less noxious than β-mercaptoethanol. The optimal levels of ferrous sulfate (500 μM) and catalase (2000 units/assay) were not significantly different in their effects on velocity or linearity of the reaction time course. Catalase was selected in anticipation of studies on iron involvement in tyrosine hydroxylase catalysis.

In these and other comparisons of assay components, only velocity and linearity were assessed. Comparative effects on other features of tyrosine hydroxylase kinetics would not have been evident.

Variation of oxygen levels. For kinetic studies involving variation of oxygen, a series of custom mixtures of oxygen in nitrogen (Air Products, San Francisco, Calif.) were used. A complete reaction mixture including all components and inhibitors, but excluding tyrosine hydroxylase, was bubbled with the desired oxygen mixture for 45 sec at a rate of 10 ml/min and capped with a rubber septum. With the reaction mixture in an ice slush, 5–10 μl of tyrosine hydroxylase were injected with a Hamilton (Hamilton Company, Reno, Nev.) repeating syringe equipped with a 7.6-cm needle which was immersed in the reaction mixture. The tyrosine hydroxylase preparation was previously degassed under vacuum with stirring for 30 min. Assay mixtures were then placed in a water bath at 37° for 6 min. At termination the mixture was cooled to 0°, uncapped, quenched with 100 μl of glacial acetic acid, and assayed as usual. Pilot experiments showed that with a bubbling rate of 10 ml/min an equilibrium O_2 tension was achieved after 30 sec of bubbling. A reaction mixture bubbled for 45 sec with pure nitrogen gave a blank value comparable to the blank values obtained in the presence of ambient O_2 and 2 mM 3-IT.

Verification of linear reciprocal functions. Since earlier studies using a crude bovine striatal supernatant and somewhat different assay conditions produced a distinctly biphasic double-reciprocal plot with 6-MPH_4 as variable substrate, and similar results indicating nonlinear kinetics of tyrosine hydroxylase have been reported, studies were designed to detect the presence of any potentially confounding kinetic features associated with the specific tyrosine hydroxylase assay system under consideration. The pilot studies were limited to variations in TYR and BH_4 levels, since complex kinetic patterns of tyrosine hydroxylase are usually manifest as a characteristic nonlinear relationship in reciprocal plots with BH_4 as variable substrate, and as classic substrate inhibition in TYR reciprocal plots. In pilot studies, the tyrosine hydroxylase preparations, assay conditions, and procedures were identical with those used in all of the studies presented below.

The experimental design relevant to Fig. 1 emphasized

precision to provide a clear indication of whether the data set exhibited any significant nonlinear character. Thus, an 11-point curve, linear in $1/BH_4$ and ranging from 10 to 330 μM BH_4, was employed. All assays were performed in triplicate, and values within triplicate determinations usually were within 1–3% of the median. The over-all precision was limited by the uncertainties in preparing the BH_4 concentration gradient. The lower levels of BH_4 (10, 11.1, and 12.5 μM) are the least reliable, since the uncertainty in concentration (± 2–5%) might result in a substantial but kinetically insignificant deviation of $1/v$ from the line.

Although no studies were designed explicitly to detect any complex kinetics associated with variation of O_2, assays with oxygen as variable substrate, as described under Materials and Methods, generally yield velocity data limited in precision and quantity due to the difficult assay procedure and four available custom O_2/N_2 mixtures. Thus, any modest curvature would not be detected. However, a principal confounding aspect of tyrosine hydroxylase kinetics with O_2 as variable substrate is inhibition by O_2 at high levels (11). Inspection of the O_2 data (Figs. 3, 4, and 7) reveals a distinct absence of inhibition at high oxygen tensions, which would be manifest as an upward curvature as the lines approach the y-axis.

Since data were carefully reviewed for any evidence suggesting complex kinetic functions and were found to follow strictly linear kinetic functions, the central kinetic studies employed abbreviated substrate concentration ranges which are unlikely to obscure any complex features.

Data reduction and representation. Data presented in Figs. 2–8 comprise subgroups of assays with a substrate concentration as the only variable. If simple saturation kinetics obtain, each subgroup of data points will be best correlated by a straight line in the double-reciprocal relationship. This is true for most of the data in question. The few subgroups with apparent curvature are probably not evidence of a more complex function, since the curvature is not systematic, and does not appear in the other subgroups of the plot.

The patterns exhibited by the experiments illustrated in Figs. 2–8 constitute the principal data base. Since the essence of data reduction and interpretation is qualitative, the data were not subjected to regression analysis. Such analyses would result in modest refinements affecting neither the patterns nor the conclusions.

Materials. 3,5-Ditritio-L-tyrosine (Amersham Corporation) was purified by adsorption to and elution from Dowex 50-H^+ resin (Sigma Chemical Company). Biopterin (Calbiochem-Boehringer, La Jolla, Calif.) was reduced with hydrogen in the presence of platinum oxide and 0.02 M HCl at ambient temperature and pressure. Reduction of biopterin under these conditions favors formation of the natural R-isomer by a factor of approximately 2 (17). Catalase, sodium succinate, 3-IT, L-tyrosine, dithiothreitol, L-dopa, and dopamine-HCl were purchased from Sigma Chemical Company. DAP was synthesized in this laboratory by a published procedure (18) or was provided by S. J. Benkovic of Pennsylvania State University (University Park, Pa).

RESULTS

The purpose, design, limitations, and relevant results of selected preliminary studies are presented under Materials and Methods to verify that the specific tyrosine hydroxylase assay system used in these kinetic studies generates data which are valid and essentially devoid of certain common complex features. In summary, a study designed to detect significant curvature in the $1/v$ versus $1/BH_4$ relationship (Fig. 1) was linear whether a crude or heparin/agarose-purified tyrosine hydroxylase was used. Curvature commonly observed in the more complex kinetics of tyrosine hydroxylase was clearly absent. Curvature characteristic of substrate inhibition was absent in $1/v$ versus 1/TYR data (data not illustrated) and was not evident in the velocity data where O_2 is variable substrate (Figs. 3, 4, and 7).

The rate of L-dopa synthesis as a function of substrate concentrations is presented in double-reciprocal plots of Figs. 2–4. With BH_4 as the variable substrate and TYR as the changing-fixed substrate, a series of lines converging to a common point below the $1/s$-axis was obtained. Thus, at ambient O_2 tension (~20.9%) the apparent Michaelis constants for BH_4 and TYR are increased with increasing concentration of the changing-fixed substrate. The limiting values of $K^{app}_{BH_4}$ and K^{app}_{TYR} (Table 1) were determined graphically. The $1/[BH_4]$ coordinate of the intersection point in Fig. 2 gave the negative reciprocal of the apparent Michaelis constant for BH_4 in the theoretical absence of TYR (19). A replot of the $1/v$ intercepts of Fig. 2 against 1/[TYR] indicated an apparent Michaelis constant for TYR at infinite BH_4. A plot of $1/v$ versus 1/[TYR] at various levels of BH_4 similarly provided the

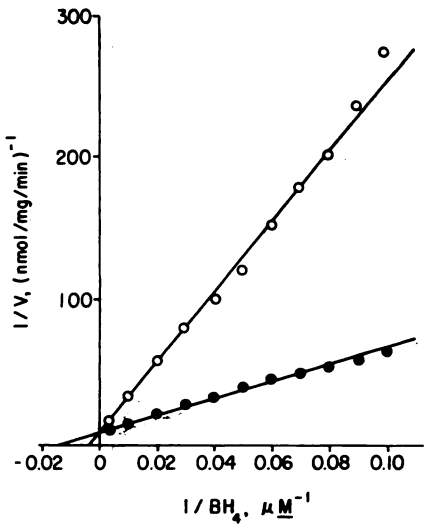

FIG. 1. *Initial velocities of tyrosine hydroxylase catalysis with variations in BH_4 concentration from 10 to 330 μM, using crude supernatant* (○) *and heparin/agarose-purified* (●) *preparations of bovine striatal tyrosine hydroxylase*

The TYR concentration was 6 μM and O_2 was at ambient tension. The velocities based on the crude supernatant were multiplied by a factor of 10 for presentation on a common y-axis. All assays were performed in triplicate and the median values were used to compute the reaction velocities.

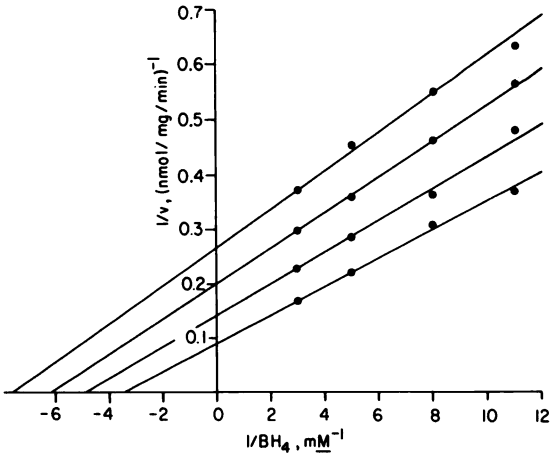

FIG. 2. *Initial velocity of tyrosine hydroxylase catalysis with variations in TYR and BH_4*

TYR concentrations were 5, 6.7, 10, and 25 μM; O_2 was at ambient tension.

apparent Michaelis constants for BH_4 at infinite TYR and for TYR in the theoretical absence of BH_4.

A double-reciprocal plot of $1/v$ versus $1/[O_2]$ at various BH_4 levels (Fig. 3) gave a series of lines converging at a common point above the $1/s$-axis, indicating that the apparent Michaelis constants for O_2 and BH_4 are decreased as the changing-fixed substrate is increased. The limiting values of K^{app}_{BH4} and $K^{app}_{O_2}$ (Table 1) were determined as described above.

When O_2 and TYR were varied (Fig. 4) a set of lines converging on or very near the $1/s$-axis was obtained, indicating the apparent Michaelis constants for O_2 and TYR to be independent of the concentration of the changing-fixed substrate.

The tyrosine analogue inhibitor 3-IT was examined with each of the three substrates as variable substrate (Table 2). With TYR as variable substrate, 3-IT exhibited linear competitive inhibition and an apparent inhibition constant K^{app}_I of 62 nM, based on a replot of slope against inhibitor concentration. When BH_4 was the

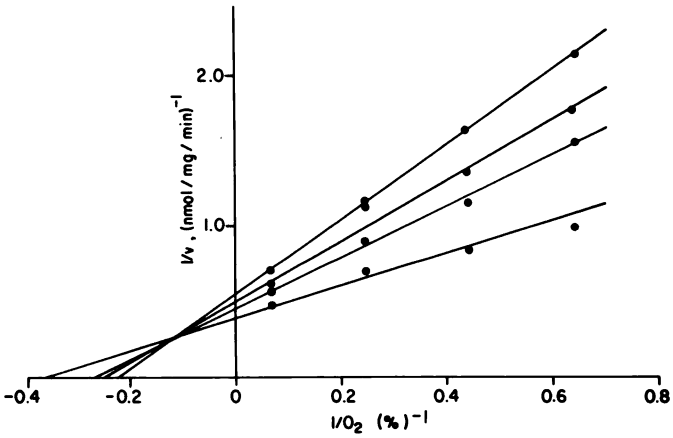

FIG. 3. *Initial velocity of tyrosine hydroxylase catalysis with variations in BH_4 and O_2*

BH_4 concentrations were 91, 125, 200, and 330 μM; the TYR concentration was 6 μM.

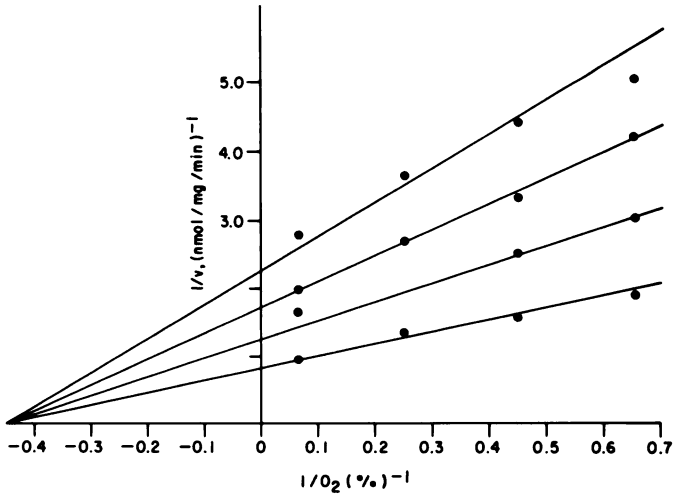

FIG. 4. *Initial velocity of tyrosine hydroxylase catalysis with variations in TYR and O_2*

TYR concentrations were 5, 6.7, 10, and 25 μM; the BH_4 concentration was 200 μM.

variable substrate, a noncompetitive pattern was observed. Both slope and $1/v$-intercept replots were linear functions of 3-IT concentration, and a common $[I]$-axis intercept indicated an apparent inhibition constant of 120 nM. 3-IT inhibition with respect to O_2 was also of the linear noncompetitive type, giving a K^{app}_I of 90 nM. The concentrations of nonvaried substrates, inhibition patterns, and apparent inhibition constants are summarized in Table 2.

The pterin analogue DAP is unable to function as a tyrosine hydroxylase cofactor[2] but does bind to tyrosine hydroxylase, as indicated by its ability to inhibit tyrosine hydroxylase catalysis. DAP was therefore examined as a pteridine cofactor analogue inhibitor against each of the

TABLE 1

Limiting values of Michaelis constants of tyrosine hydroxylase for TYR, BH_4, and O_2

Variable substrate (concentration range)	K_m^{app}	Extrapolated or actual concentrations of other substrates		
		BH_4	TYR	O_2
		μM	μM	%
TYR (5–25 μM)	25 μM[a]	∞	—	20
	2.8 μM[b]	0	—	20
	20 μM	200	—	All levels
BH_4(91–330 μM)	476 μM[a]	—	∞	20
	59 μM[b]	—	0	20
	97 μM[a]	—	6	∞
O_2 (1.57–15.6%)	2.22%[b]	200	0	—
	1.43%[a]	∞	6	—
	10.0%[b]	0	6	—

[a] K_m^{app} was taken as the negative reciprocal of the x-intercept in a replot of $1/v$-intercepts versus 1/[changing-fixed substrate].

[b] K_m^{app} was taken as the negative reciprocal of the abscissa value of the common intersection point for lines in the double-reciprocal plot.

[2] W. P. Bullard, unpublished observation.

TABLE 2

Dead-end and product inhibition of tyrosine hydroxylase

Inhibitor	Inhibitor concentration range	Variable substrate[a]	No. of data points[b]	Concentration of nonvaried substrates			Type of inhibition[c]	Apparent inhibition constant[d]
				TYR	BH_4	O_2		
	μM			μM	μM	%		
3-IT	0.04–0.16	TYR	20	—	200	20	LC	62 nM
	0.02–0.08	BH_4	20	6	—	20	LN	120 nM
	0.04–0.16	O_2	16	6	200	—	LN	90 nM
DAP	250–1000	TYR	20	—	300	20	LN	450 μM
	250–1000	BH_4	20	6	—	20	LC	250 μM
	250–1000	O_2	16	6	200	—	LN	290 μM
Dopamine	15–60	BH_4	20	6	—	20	LC	20 μM
	15–60	TYR	20	—	300	20	LN	35 μM
L-Dopa	50–200	TYR	48	5–25[e]	91–330	20	LN[f]	33 μM
	50–200	BH_4	48	5–25[e]	91–330	20	LN[f]	34 μM
	75–300	O_2	20	6	200	—	LN	100 μM

[a] TYR was varied from 5 to 25 μM; BH_4 from 91 to 330 μM; and O_2 from 1.57 to 15.6%.
[b] Each point represents the average of duplicate determinations.
[c] All slope and $1/v$-intercepts were linear functions of inhibitor concentration. LC, linear competitive; LN, linear noncompetitive.
[d] Apparent inhibition constants were graphically determined from slope or slope and $1/v$-intercept replots of double-reciprocal plots.
[e] A complete matrix using four levels each of L-dopa, BH_4, and TYR was examined.
[f] Parabolic slope replots were occasionally observed in other L-dopa inhibition studies.

three substrates. DAP inhibition with respect to BH_4 as variable substrate was competitive, and a replot exhibited a linear dependence of slope on inhibitor concentration. The slope replot indicated a K^{app}_I of 450 μM. With TYR or O_2 as variable substrate, simple linear noncompetitive inhibition obtained. The apparent inhibition constants were 250 μM and 290 μM, respectively, under the conditions used.

Dopamine (3,4-dihydroxyphenylethylamine), the principal catecholamine neurotransmitter in striatum, has been established as an inhibitor of tyrosine hydroxylase (1). Inhibition of tyrosine hydroxylase by dopamine was examined with BH_4 and TYR as variable substrates. The instability of dopamine with respect to oxidation under the reaction conditions and time periods required for oxygen variation studies precluded dopamine inhibition studies with O_2 as variable substrate.[2] Inhibition by dopamine was linearly competitive when BH_4 was varied and linear noncompetitive when TYR was varied. The apparent inhibition constants and reaction conditions appear in Table 2.

The immediate products of tyrosine hydroxylase catalysis are L-dopa and a form of dihydropterin (BH_2). The actual identity of the dihydropterin product remains obscure, and product inhibition studies with this product were precluded. L-dopa, as a product inhibitor, was examined under a range of reaction conditions. L-dopa inhibition with TYR (Fig. 5), BH_4 (Fig. 6), or O_2 (Fig. 7) as the variable substrate exhibited noncompetitive inhibition. In all cases, linear slope and intercept replots were obtained. In certain individual experiments (the data of Fig. 5 included), the slope replots were parabolic, and in the case of L-dopa inhibition with O_2 as variable substrate, the slope replot exhibited a relatively small de-

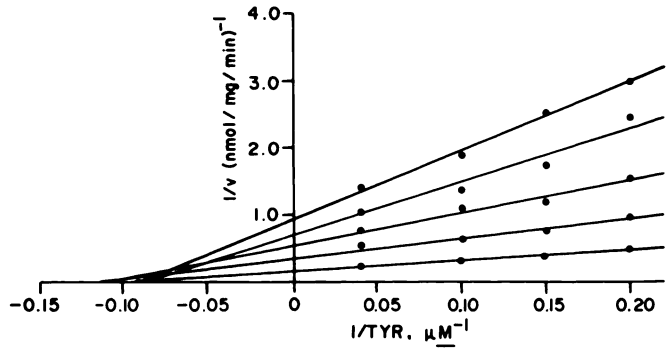

FIG. 5. *L-Dopa inhibition of tyrosine hydroxylase with respect to TYR*

L-Dopa concentrations were 0, 50, 100, 150, and 200 μM; BH_4, 200 μM; and O_2, ambient tension.

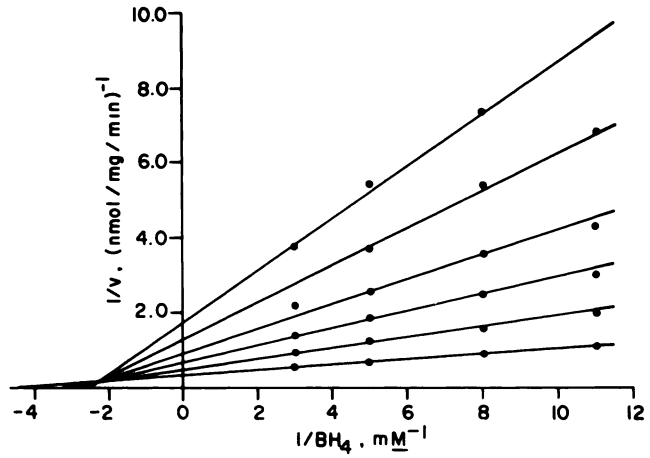

FIG. 6. *L-Dopa inhibition of tyrosine hydroxylase with respect to BH_4*

L-Dopa concentrations were 0, 50, 100, 150, and 200 μM; TYR, 6 μM; and O_2, ambient tension.

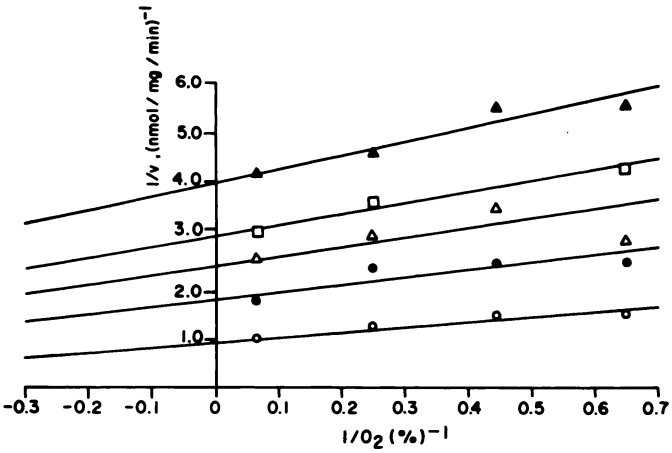

FIG. 7. *L-Dopa inhibition of tyrosine hydroxylase with respect to O_2*

L-Dopa concentrations were 0, 75, 150, 225, and 300 μM; BH_4, 200 μM; and TYR, 6 μM.

pendence on inhibitor concentration, as can be discerned by inspection of Fig. 7.

In a separate experiment, L-dopa inhibition was examined with BH_4 as variable substrate at four different TYR levels, under ambient O_2 tension. A series of primary double-reciprocal plots of L-dopa inhibition against BH_4 were constructed for each of four TYR levels. The $1/v$-axis intercepts of the plots, representing an infinite BH_4 concentration, were then plotted against 1/[TYR] to give the inhibition plot (Fig. 8) of L-dopa with TYR as the variable substrate at infinite BH_4 concentration. The resulting inhibition pattern is distinctly noncompetitive, with linear slope and $1/v$-intercept replots. The same data were used to construct a plot representing L-dopa inhibition with BH_4 as the variable substrate with an infinite TYR concentration (data not shown). The resulting plot exhibited noncompetitive inhibition.

DISCUSSION

The preliminary data and results presented in Figs. 1–8 are devoid of the nonlinear components that are characteristic of substrate inhibition, cooperativity, catalysis by multiple enzyme forms, or any apparent complex kinetics often observed in tyrosine hydroxylase catalysis. The absence of such features provides an opportunity to examine the catalytic mechanism using the classic models for simple saturation kinetics.

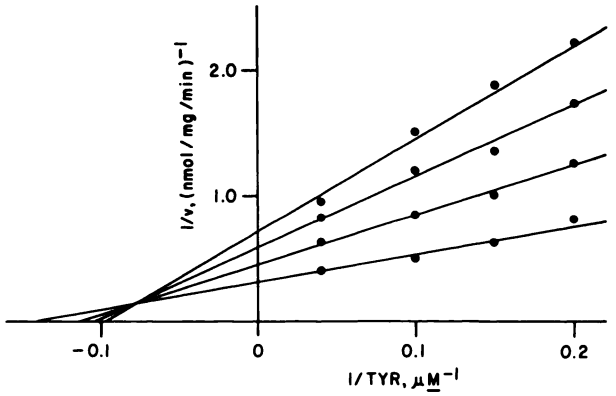

FIG. 8. *L-Dopa inhibition of tyrosine hydroxylase catalysis at infinite BH_4 concentration*

The inhibition of L-dopa (50, 100, 150, and 200 μM) with BH_4 as the variable substrate (91, 125, 200, and 330 μM) was examined at four different TYR levels (5, 6.7, 10, and 25 μM). The $1/v$-intercepts of the primary double-reciprocal plots were plotted against 1/[TYR].

The velocity of tyrosine hydroxylase-catalyzed L-dopa synthesis as a function of BH_4, TYR, and O_2 concentrations (Figs. 2–4) indicates that, taking any two reactants as variable and changing-fixed substrates, the double-reciprocal plot will exhibit a series of convergent straight lines. The addition of all three substrates to the enzyme therefore occurs in a series of reversible steps prior to the occurrence of any irreversible catalytic or product release steps (20). Thus a sequential mechanism is apparently operating resulting in the formation of a quaternary complex, and a Ping Pong (20) mechanism is excluded.

The complex relationship between apparent Michaelis constants and concentrations of nonvaried substrates (Table 1) is difficult to interpret in the absence of evidence for a rapid-equilibrium or a steady-state addition of substrates to tyrosine hydroxylase. The most pronounced effect is seen in the relationship between BH_4 and TYR in which increasing the concentration of either reactant decreases the enzyme's apparent affinity for the other. Increasing O_2 tension moderately increases the enzyme's apparent affinity for BH_4 and vice versa. There is no discernible effect of O_2 tension on the enzyme's affinity for TYR, and similarly the affinity for O_2 is unaffected by TYR concentration.

Dead-end inhibition by 3-IT and DAP (Table 2) provides information on the order of substrate binding. The competitive inhibition pattern observed in 3-IT inhibition with TYR as the variable substrate suggests that 3-IT is recognized by tyrosine hydroxylase as an analogue of TYR and that 3-IT binds to the same enzyme form as TYR. The noncompetitive inhibition of 3-IT with respect to BH_4 requires that BH_4 be able to bind to tyrosine hydroxylase after 3-IT and presumable, therefore, after TYR in the normal catalytic sequence. If 3-IT were required to bind after BH_4, the slope term in the double-reciprocal plot would vanish and uncompetitive inhibition would result (20). A similar rationale applied to the competitive inhibition by DAP with respect to BH_4, and noncompetitive inhibition by DAP with respect to TYR suggests that TYR is able to bind after BH_4 is bound. The dead-end inhibition data thus indicate that either BH_4 or TYR can bind first and that binding with respect to these two substrates is random. Data on product inhibition by L-dopa corroborates this conclusion (*vide infra*).

The order for O_2 addition in the sequence is not completely established from the data. However, some possible kinetic mechanisms can be eliminated. If O_2 were the obligatory first reactant to bind, followed by addition of TYR and BH_4, inhibition by the substrate and cofactor analogues 3-IT and DAP with O_2 as variable substrate would be strictly uncompetitive. The data for 3-IT and DAP inhibition with O_2 as variable substrate (Table 2) exhibit noncompetitive inhibition, indicating that O_2 can bind after either BH_4 or TYR. A mechanism featuring O_2 addition as an obligatory last step in substrate addi-

tion may give rise to either of two rate equations depending upon whether O_2 addition occurs in a rapid equilibrium or steady-state step (19). In the rapid equilibrium case, the equation predicts that, at infinite O_2 concentrations, the velocity dependence on BH_4 and TYR would vanish. Thus, variation of O_2 at different fixed concentrations of either BH_4 or TYR would exhibit an intersection of the lines on the $1/v$-axis. Figures 3 and 4 demonstrate intersection to the *left* of the $1/v$-axis. The rate equation for random rapid-equilibrium addition of TYR and BH_4 followed by the steady-state addition of O_2 is not readily distinguishable from the totally random rapid-equilibrium case (19), and the data are compatible with either. The data are also compatible with a mechanism featuring random addition of BH_4 and TYR with O_2 binding only after BH_4 is bound, provided that the over-all addition of substrates is not strictly in rapid equilibrium relative to the interconversion of the central complexes. The observation that BH_4 appears to bind prior to O_2 in the similar conversion of phenylalanine to TYR by phenylalanine hydroxylase[3] argues for the latter mechanism, but, for tyrosine hydroxylase, this aspect of the mechanism for substrate addition remains to be established.

The two products of the tyrosine hydroxylase-catalyzed reaction are L-dopa and a dihydropterin (BH_2). The exact structure of the dihydropterin product has not been established, and product inhibition studies have therefore been restricted to tyrosine hydroxylase inhibition by L-dopa. The data of Figs. 5–8 provide information on the order of product release steps as well as substrate addition order. Release of products from tyrosine hydroxylase might occur in an ordered or rapid-equilibrium random fashion. Regardless of the order of substrate addition, both the rapid-equilibrium release and ordered release with BH_2 preceding L-dopa predict that L-dopa would inhibit competitively with respect to at least one of the three reaction substrates. The data for L-dopa inhibition show L-dopa to be noncompetitive with respect to all three reactants and are therefore compatible only with an ordered mechanism for product release in which L-dopa is the first product released.

An ordered release of products with L-dopa preceding BH_2, when combined with an ordered addition of BH_4 and TYR, would predict that L-dopa inhibition with the first substrate as variable will exhibit an uncompetitive pattern when the second substrate is saturating (20). However, Fig. 8 shows that L-dopa inhibition against TYR remains noncompetitive at an infinitely high BH_4 concentration. A similar plot of L-dopa inhibition against BH_4 at infinite TYR (data not shown) is also noncompetitive. The persistent slope effects in the above data corroborate the dead-end inhibition data by indicating a random order of addition for TYR and BH_4.

Dead-end inhibition of tyrosine hydroxylase by dopamine and a variety of catechols other than L-dopa has been reported to be competitive with respect to the pteridine cofactor and noncompetitive with respect to TYR (16, 21). The data on dopamine inhibition (Table 2) are in agreement with previous reports, but should be considered against a background of L-dopa inhibition results. As a product analogue, dopamine might be expected to bind to the tyrosine hydroxylase form that binds the product, L-dopa. Product inhibition indicates that L-dopa binds to the enzyme form to which BH_2 is bound, but not in the absence of BH_2. Binding of dopamine as a dead-end inhibitor at the L-dopa site would result in uncompetitive inhibition and is therefore excluded. Dopamine is required by the data to bind instead to the same enzyme form to which BH_4 binds, but the basis for the differences between dopamine and L-dopa binding remains obscure.

The conclusion that dopamine and L-dopa bind to different kinetic forms of tyrosine hydroxylase provides a possible explanation for the fact that occasionally slope replots of L-dopa inhibition data exhibit a parabolic curvature. In the proposed scheme, L-dopa would exert its principal inhibitory effect by binding to the $E \cdot BH_2$ complex. If, in addition, L-dopa bound in the same fashion as dopamine, i.e., as a dead-end inhibitor, parabolic replots would result (20).

The relatively uncomplicated substrate and inhibition kinetics of the bovine striatal tyrosine hydroxylase reveal some apparent constraints on the order of events comprising the over-all catalytic sequence. Compared with a random Ter Bi mechanism, the tyrosine hydroxylase catalytic sequence is more highly organized, with an obligatory order for product release and perhaps partial ordering with respect to BH_4 and O_2 addition steps. The ordered mechanism may underlie some of the complex kinetics of tyrosine hydroxylase catalysis. For example, the more complex kinetic behavior of adrenal tyrosine hydroxylase (12) features substrate inhibition by TYR under certain conditions. An ordered product release commencing with L-dopa produces the $E \cdot BH_2$ complex which might readily bind the structurally similar TYR to form an $E \cdot TYR \cdot BH_2$ dead-end complex, resulting in substrate inhibition. In this hypothetical mechanism the stereochemistry of the pterin might influence the binding of TYR to $E \cdot BH_2$, as was experimentally observed. The details relating the proposed scheme to complex kinetic behavior of tyrosine hydroxylase remain to be established, but should provide a unique perspective for further characterization of the catalytic mechanism.

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REFERENCES

1. Nagatsu, T., M. Levitt, and S. Udenfriend. Tyrosine hydroxylase: the initial step in norepinephrine biosynthesis. *J. Biol. Chem.* **239**:2910–2917 (1964).
2. Levitt, M., S. Spector, A. Sjoerdsma, and S. Udenfriend. Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. Exp. Ther.* **148**:1–8 (1965).
3. Ikeda, M., L. A. Fahien, and S. Udenfriend. A kinetic study of bovine adrenal tyrosine hydroxylase. *J. Biol. Chem.* **241**:4452–4456 (1966).
4. Joh, T. H., R. Kapit, and M. Goldstein. A kinetic study of particulate bovine adrenal tyrosine hydroxylase. *Biochim, Biophys. Acta* **171**:378–389 (1969).
5. Musacchio, J. M., C. A. McQueen, and G. L. Craviso. Tyrosine hydroxylase: subcellular distribution and molecular and kinetic characteristics of the different enzyme forms, in *New Concepts in Neurotransmitter Regulation* (A. J. Mandell, ed.). Plenum Press, New York, 69–88 (1973).
6. Musacchio, J. M., and G. L. Craviso. Properties of tyrosine hydroxylase, in *Frontiers in Catecholamine Research* (E. Usdin and S. Snyder, eds.). Per-

[3] S. J. Benkovic, personal communication.

gamon Press, New York, 47–52 (1973).
7. Kaufman, S. Cofactors of tyrosine hydroxylase, in *Frontiers in Catecholamine Research* (E. Usdin and S. Snyder, eds.). Pergamon Press, New York, 53–60 (1973).
8. Weiner, N., F.-L. Lee, E. Dreyer, and E. Barnes. The activation of tyrosine hydroxylase in noradrenergic neurons during acute nerve stimulation. *Life Sci.* **22**:1197–1216 (1978).
9. Vulliet, P. R., T. A. Langan, and N. Weiner. Tyrosine hydroxylase: a substrate of cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **77**:92–96, (1980).
10. Lloyd, T., and S. Kaufman. The stimulation of partially purified bovine caudate tyrosine hydroxylase by phosphatidyl-L-serine. *Biochem. Biophys. Res. Commun.* **59**:1262–1269 (1974).
11. Fisher, D. B., and S. Kaufman. The inhibition of phenylalanine and tyrosine hydroxylases by high oxygen levels. *J. Neurochem.* **19**:1359–1365 (1972).
12. Oka, K., T. Kato, T. Sugimoto, S. Matsuura, and T. Nagatsu. Kinetic properties of tyrosine hydroxylase with natural tetrahydrobiopterin as cofactor. *Biochim. Biophys. Acta* **661**:45–53 (1981).
13. Kato, T., K. Oka, T. Nagatsu, T. Sugimoto, and S. Matsuura. Effects of structures of tetrahydropterin cofactors on tyrosine hydroxylase. *Biochim. Biophys. Acta* **612**:226–232 (1980).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
15. Nagatsu, T., M. Levitt, and S. Udenfriend. A rapid and simple radio assay for tyrosine hydroxylase activity. *Anal. Biochem.* **9**:122–126 (1964).
16. Udenfriend, S., P. Zaltzman-Nirenberg, and T. Nagatsu. Inhibitors of purified beef adrenal tyrosine hydroxylase. *Biochem. Pharmacol.* **14**:837–845 (1965).
17. Bailey, S. W., and J. E. Ayling. Separation and properties of the 6-diastereoisomers of *l-erythro*-tetrahydrobiopterin and their reactivities with phenylalanine hydroxylase. *J. Biol. Chem.* **253**:1598–1605 (1978).
18. Moad, G., C. L. Luthy, and S. J. Benkovic. The mechanism of oxidation of 6-methyl-5-carba-5-deazatetrahydropterin: evidence for the involvement of a 4a-adduct in the oxidation of tetrahydropterins. *Tetrahedron Lett.* **26**:2271–2274.
19. Segel, I. H. *Enzyme Kinetics*. Wiley-Interscience, New York (1975).
20. Cleland, W. W. Steady state kinetics, in *The Enzymes* (P. D. Boyer, ed.). Academic Press, New York, 1–65 (1970).
21. Levitt, M., J. W. Gibb, J. W. Daly, M. Lipton, and S. Udenfriend. A new class of tyrosine hydroxylase inhibitors and a simple assay of inhibition *in vivo*. *Biochem. Pharmacol.* **16**:1313–1321 (1967).

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