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Studies of the Rate-Limiting Step in the Tyrosine Hydroxylase Reaction: Alternate Substrates, Solvent Isotope Effects, and Transition-State Analogues†

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ABSTRACT: Tyrosine hydroxylase catalyzes the formation of dihydroxyphenylalanine from tyrosine, utilizing a tetrahydropterin and molecular oxygen as cosubstrates. Several approaches were taken to examining the identity of the rate-limiting step in catalysis. Steady-state kinetic parameters were determined with a series of ring-substituted phenylalanines. The V_{max} value was unchanged with substrates ranging in reactivity from tyrosine to 4-fluorophenylalanine. Neither 4-pyridylalanine *N*-oxide, a model of tyrosine phenoxide, nor 4-hydroxy-3-pyridylalanine *N*-oxide or α-amino-3-hydroxy-4-pyridone-1-propionic acid, models of a hydroxycyclohexadienone intermediate, was an effective inhibitor. There was no solvent isotope effect on either the V_{max} or the V/K_{Tyr} value. These results establish that no chemistry occurs at the amino acid in the rate-limiting step and no exchangeable proton is in flight in the rate-limiting step. The results are consistent with a model in which the slow step in catalysis is formation of the hydroxylating intermediate.

The enzyme tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of the catecholamine neurotransmitters, the hydroxylation of tyrosine to form dihydroxyphenylalanine (Kaufman & Kaufman, 1985) (Scheme I). This enzyme is one of several poorly understood aromatic amino acid hydroxylases which utilize tetrahydropterins as substrates and contain iron in the active site; phenylalanine hydroxylase and tryptophan hydroxylase are other members of this group (Benkovic, 1980; Shiman, 1985). The actual mechanism of hydroxylation by these enzymes and even the identity of the hydroxylating intermediate are unknown. The pterin product released from the enzyme during catalysis has been shown to be a 4a-hydroxytetrahydropterin for both tyrosine hydroxylase

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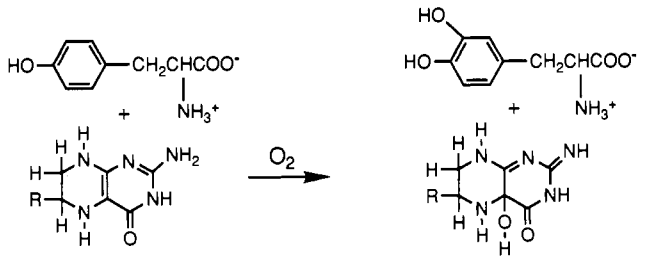
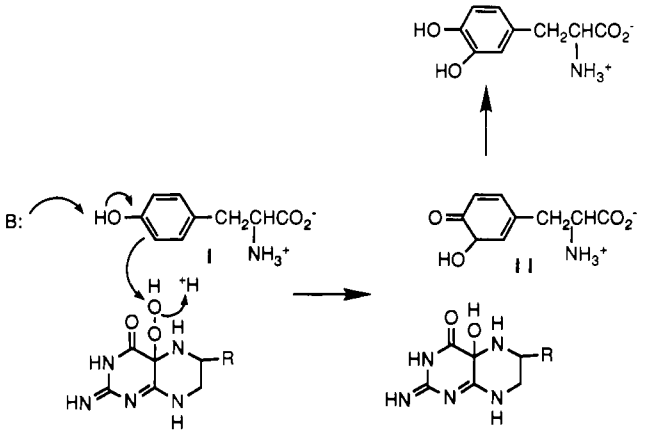
Scheme I



Scheme II



(Dix et al., 1987; Haavik & Flatmark, 1987) and phenylalanine hydroxylase (Lazarus et al., 1981). On this basis, several authors have suggested that a 4a-peroxytetrahydropterin is either the hydroxylating species or an intermediate in its formation (Shiman, 1985; Benkovic, 1980; Kaufman & Kaufman, 1985). With phenylalanine hydroxylase, enzymatic turnover of substrates which are not hydroxylated results not in the production of a 4a-hydroxytetrahydropterin but in the direct formation of dihydropterin and hydrogen peroxide (Dix & Benkovic, 1985). Such a result is consistent with partitioning of a peroxytetrahydropterin intermediate.

Flavoprotein hydroxylases carry out a reaction similar to that catalyzed by tyrosine hydroxylase, the ortho hydroxylation of a phenol. A 4a-hydroxyflavin is known to be formed as an intermediate in the reaction of the flavoprotein phenol hydroxylases. These enzymes are also known to form a 4a-peroxyflavin prior to hydroxylation (Entsch et al., 1976). The latter species has been proposed to be the actual hydroxylating intermediate, although the mechanism of hydroxylation is still unsettled (Taylor & Massey, 1990). A frequently proposed mechanism involves attack of the aromatic ring of the substrate on the electrophilic flavin or pterin peroxide; this would be assisted by the electron-donating ability of the phenolic group. The mechanism of Scheme II is analogous to proposals for the mechanism of phenol hydroxylation by flavoprotein hydroxylases (Detmer & Massey, 1985). The hydroxycyclohexadienone II has been explicitly proposed as an intermediate in the reaction catalyzed by tyrosine hydroxylase (Guroff et al., 1967).

We have recently determined the steady-state kinetic mechanism of rat tyrosine hydroxylase (Fitzpatrick, 1991). The mechanism is ordered, with substrates binding in the order tetrahydropterin, oxygen, and then tyrosine. There is no burst of DOPA[1] production in the first turnover, establishing that product release must be rapid and that chemistry is probably rate-limiting. No primary isotope effect is seen on any kinetic parameter when [3,5-$^2H_2$]tyrosine is used as the substrate for tyrosine hydroxylase, so that carbon–hydrogen bond cleavage does not occur in a slow step. However, the lack of an isotope effect is consistent with most mechanisms involving attack by an electrophilic oxygen species. It does not allow one to determine which chemical events are rate-limiting in catalysis. This report describes further probes of the identity of the rate-limiting step and the catalytic mechanism of this important enzyme.

Experimental Procedures

[3,5-^{3}H]Tyrosine was purchased from either New England Nuclear or Amersham Corp.; it was purified before use by the method of Ikeda et al. (1966). The concentrations of stock solutions of tyrosine for kinetic isotope effect measurements were determined spectrophotometrically by using a value of ϵ_{274} = 1.39 mM^{-1} cm^{-1} (Fasman, 1989). 6-Methyltetrahydropterin was synthesized as described previously (Fitzpatrick, 1988). 6-Methyl-5-deazatetrahydropterin was synthesized by the method of Moad et al. (1979). (6*R*)-Tetrahydrobiopterin was purchased from Research Biochemicals, Inc. The concentrations of tetrahydropterin solutions were determined from the absorbance at 266 nm in 2 M perchloric acid by using extinction coefficients of 17.8 mM^{-1} cm^{-1} for 6-methyltetrahydropterin (Shiman et al., 1971) and 18 mM^{-1} cm^{-1} for tetrahydrobiopterin (Hill et al., 1988). Stock solutions were made up fresh daily in 5 mM HCl. Solutions of ferrous ammonium sulfate were made up as 1 mM stocks at pH 3 and stored at −20 °C until thawed for use; they were not reused once thawed. α-Amino-3-hydroxy-4-pyridone-1-propionic acid was purchased from Sigma. Pyridylalanine *N*-oxide was synthesized by the method of Sullivan et al. (1968). 4-Hydroxy-3-pyridylalanine *N*-oxide was synthesized by the method of Tilley et al. (1979). 4-Pyridylalanine and 3-pyridylalanine were synthesized by the method of Bixler and Niemann (1958). 4-Fluoro-3-pyridylalanine and 4-hydroxy-3-pyridylalanine were synthesized by the method of Sullivan et al. (1971). 4-Bromo-3-pyridylalanine was synthesized by the method of Sullivan and Norton (1971). The small amounts of tyrosine that contaminate commercial preparations of 4-methoxyphenylalanine were removed by preparative HPLC. All other substrates were also checked for the presence of tyrosine.

Catalase was from Boehringer-Mannheim. Sheep dihydropterin reductase was from Sigma. Rat tyrosine hydroxylase expressed in insect tissue culture cells was purified as described previously (Fitzpatrick et al., 1990). Bovine adrenal tyrosine hydroxylase was purified to between 50 and 80% homogeneity by the method of Oka et al. (1982). Concentrations of tyrosine hydroxylase were determined by using an $\epsilon_{280}^{1\%}$ value of 10.4 (Haavik et al., 1988) and a subunit molecular weight of 56 000.

Initial rates with alternate substrates were determined in a coupled assay by following at 340 nm the NADH-linked reduction of the quinonoid dihydropterin product by dihydropterin reductase. Assays contained variable amounts of both amino acid and tetrahydrobiopterin in 5 μM ferrous ammonium sulfate, 75 μg/mL catalase, 200 μM NADH, 0.45 unit/mL sheep dihydropterin reductase, 50 mM sodium acetate, 50 mM MES, and 0.1 M Tris-HCl, pH 6.5, at 30 °C. Assays were initiated by the addition of enzyme after the autoxidation of the tetrahydropterin had stabilized. The observed rates were corrected for the background due to tetrahydropterin autoxidation.

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; DOPA, dihydroxyphenylalanine; 6-MPH_4, 6-methyltetrahydropterin; BH_4, (6*R*)-tetrahydrobiopterin.

To measure the rate of DOPA formation from 3-hydroxyphenylalanine, a modification of the colorimetric procedure of Arnow (1937) was used as previously described (Fitzpatrick, 1991). Conditions identical with those of the linked assay were used, except that NADH and dihydropterin reductase were omitted. The assays were initiated by the addition of 6-methyltetrahydropterin because of the lack of a recycling system.

For the determination of inhibition constants with bovine tyrosine hydroxylase, the assay was based on the release of tritium from [3,5-^{3}H]tyrosine as previously described (Fitzpatrick, 1988). Standard assay conditions were 75 μg/mL catalase, 14 mM β-mercaptoethanol, and 50 mM sodium pyrophosphate, pH 6, at 37 °C, in a volume of 1 mL. Assays were started by adding either 6-methyltetrahydropterin or enzyme. After 5 min at 37 °C, the reaction was quenched by adding 0.05 mL of acetic acid, and the tritiated water was separated on a Dowex column. To test for time-dependent inhibition, the enzyme was incubated with the inhibitor at 37 °C in 50 mM Tris-HCl, pH 7.0 or 7.9, and aliquots were withdrawn over 1–2 h for assays.

To determine solvent isotope effects, initial rates were determined in H_2O and D_2O by following the loss of tritium from [3,5-^{3}H]tyrosine (Fitzpatrick, 1989). All solutions except the enzyme were made up in H_2O or D_2O. The amount of enzyme added was less than 1% of the total volume. The V_{max} and V/K_{Tyr} values were determined with 5–120 μM tyrosine, 250 μM oxygen, and 550 μM 6-methyltetrahydropterin in 50 mM MES, 50 mM Tris–acetate, 12 μM ferrous ammonium sulfate, and 90 μg/mL catalase, at 30 °C.

Steady-state kinetic data were fit to the relevant programs of Cleland (1979), using programs adapted for use on a Macintosh microcomputer by Dr. James Robertson of Pennsylvania State University. The specific equations used are indicated in the legends to the tables and figures.

Results

Steady-State Kinetics with Alternate Substrates. Analysis of the effects of altering the reactivities of substrates upon kinetic parameters can be a powerful probe of enzyme mechanisms. Accordingly, steady-state kinetic parameters were determined with several substituted phenylalanines as substrates for tyrosine hydroxylase. The physiological substrate tetrahydrobiopterin (BH_4) was used as cosubstrate because its lower K_M value allowed lower levels of tetrahydropterin to be used compared to those required with 6-methyltetrahydropterin, decreasing the background rates due to tetrahydrobiopterin autoxidation. Also, with BH_4 as substrate under the conditions of these experiments (pH 6.5, 30 °C), oxygen is saturating when tyrosine is the substrate (Fitzpatrick et al., 1990). The assay that was used followed the rate of production of dihydrobiopterin, to ensure the total rate of turnover was measured, independently of whether hydroxylation of the amino acid substrate occurred.

In all cases, the observed rates depended directly on the enzyme concentration. No turnover was seen in the absence of amino acid or in the presence of saturating levels of the competitive inhibitor 3-iodotyrosine, indicating that the measured rates were due to catalysis by tyrosine hydroxylase. The rates of DOPA production from 3-hydroxyphenylalanine measured by a colorimetric method and from tyrosine measured by tritium release from [3,5-^{3}H]tyrosine were indistinguishable from those of Table I (results not shown).

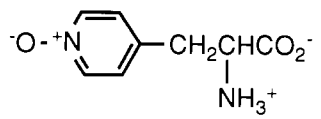
The kinetic parameters with a number of substituted phenylalanines which vary widely in reactivity are summarized in Table I. The data with all of the substrates except tryptophan were fit best by a sequential kinetic mechanism; the data with tryptophan as substrate fit slightly better to a ping-pong mechanism. This is consistent with previous studies of the kinetics with tyrosine as substrate. Substrate inhibition was seen at high levels of all of the amino acids except 4-methoxyphenylalanine; the limited solubility of that substrate made it impossible to do assays at concentrations above 2 mM. In addition, no turnover was detected with 3-pyridylalanine.

Table I: Steady-State Kinetic Parameters for Alternate Substrates for Rat Tyrosine Hydroxylase[a]

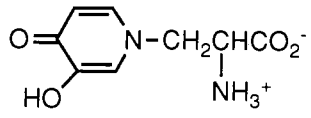
substrate	V_{max} (min^{-1})	K_{aa} (μM)	K_{BH_4} (μM)
tyrosine	92.4 ± 10.7	10.8 ± 4.4	17.7 ± 2.5
4-aminophenylalanine	115 ± 5.8	36.8 ± 10.2	32.2 ± 8.1
4-methoxyphenylalanine	65 ± 6.1	458 ± 84	14.3 ± 2.4
phenylalanine	111 ± 6.6	49.3 ± 10.9	15.2 ± 4.1
4-fluorophenylalanine	83 ± 4.8	11.8 ± 2.2[b]	25.1 ± 2.6
3-hydroxyphenylalanine	83.9 ± 7.4	219 ± 41[b]	13.1 ± 2.8
3-fluorophenylalanine	118 ± 17	23.5 ± 8[b]	29.9 ± 9.2
tryptophan	27.4 ± 6.6	210 ± 63	19.6 ± 6.6

[a] Initial velocities were determined as the rate of dihydropterin production with variable amounts of both amino acid and tetrahydrobiopterin in 5 μM ferrous ammonium sulfate, 75 μg/mL catalase, 200 μM NADH, 0.45 unit/mL sheep dihydropterin reductase, 50 mM sodium acetate, 50 mM MES, and 0.1 M Tris-HCl, pH 6.5, at 30 °C. The data were fit to $v = VAB/(AB + AK_b + BK_a + K_{ia}K_b)$ with the exception of the results with tryptophan which fit best to $v = VAB/(AB + AK_b + BK_a)$. [b] Determined with the racemic mixture, assuming that only the L-isomer is a substrate.

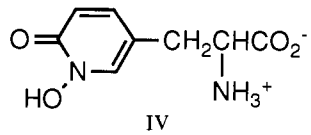
Transition-State Analogues. As a test of the mechanism proposed in Scheme II, several compounds similar to the proposed intermediates were tested as inhibitors. The proposed removal of the phenolic proton of tyrosine, a reasonable way of further activating the aromatic ring, predicts that the phenoxide will bind more tightly to the enzyme than the protonated form of tyrosine. 4-Pyridylalanine *N*-oxide (III)



III



V



IV

was tested as a mimic of the proposed phenoxide. The preferred tautomer of both 4-hydroxy-3-pyridylalanine *N*-oxide (IV) and 3-[N-(3′-HO-4′-pyridone)]-2-aminopropionic acid (V) is the keto species (Katritzky & Lagowski, 1976), so they were tested as analogues of the proposed hydroxycyclohexadienone species II. Bovine adrenal tyrosine hydroxylase was used for these experiments because IV and V were not stable in the presence of ferrous ammonium sulfate, a necessary component of the assay for the recombinant rat enzyme. To

Table II: Inhibition Constants for Bovine Adrenal Tyrosine Hydroxylase[a]

inhibitor	K_{is} (mM)	K_{ii} (mM)
3-pyridylalanine	0.78 ± 0.11[b]	
4-pyridylalanine	1.1 ± 0.087[b]	
4-pyridylalanine *N*-oxide (III)	≫4[c]	
4-fluoro-3-pyridylalanine	≫2[c]	
4-fluorophenylalanine	0.34 ± 0.008[b]	
phenylalanine	0.13 ± 0.02[b]	
4-bromo-3-pyridylalanine	0.47 ± 0.28[b]	
4-hydroxy-3-pyridylalanine	≫4[c]	
4-chloro-3-pyridylalanine *N*-oxide	≫4[c]	
4-hydroxy-3-pyridylalanine *N*-oxide (IV)	2.7 ± 1.4[d]	1.3 ± 0.26
DOPA	0.18 ± 0.019[d]	0.17 ± 0.008
α-amino-3-hydroxy-4-pyridone-1-propionic acid (V)		0.44 ± 0.075[e]

[a] Conditions: 20–200 μM tyrosine, 14 mM β-mercaptoethanol, 65 μg/mL catalase, 0.205 mM oxygen, 1 mM 6-MPH$_4$, and 50 mM sodium pyrophosphate, pH 6.0, at 37 °C. [b] The data were fit to $v = VS/[S + K_M(1 + I/K_{is})]$. [c] No inhibition was seen. The value is the highest level of inhibitor tested. [d] The data were fit to $v = VS/[S(1 + I/K_{ii}) + K_M(1 + I/K_{is})]$. [e] The data were fit to $v = VS/[S(1 + I/K_{ii}) + K_M]$.

ascertain the effect upon binding of substitution of a pyridyl ring for a phenyl ring, several pyridylalanines were also tested as reversible inhibitors versus tyrosine. Such a substitution decreased the affinity of the enzyme for the inhibitor by about an order of magnitude (Table II). However, with all three compounds, only very weak or no inhibition was seen, and the inhibition was more characteristic of DOPA analogues than amino acids. Also, no time-dependent inhibition was seen with either III or IV, either alone or when 6-methyl-5-deazatetrahydropterin was added as a tetrahydropterin analogue (results not shown).

Solvent Isotope Effects. The steady-state kinetic parameters of tyrosine hydroxylase are pH dependent (Fitzpatrick et al., 1990). The V_{max}–pH profile is bell-shaped, suggesting the possibility of acid or base catalysis. In addition, formation of a peroxypterin and hydroxylation of tyrosine must involve several proton transfers. To determine if transfer of a solvent-exchangeable proton occurred in a slow step, solvent isotope effects on the V_{max} and V/K_{Tyr} values were determined. Initial rates were determined with saturating concentrations of 6-methyltetrahydropterin and oxygen at varied tyrosine concentrations from pH 6 to 8 or pD 6.6 to 8.6 (Figure 1). Direct comparison of the V_{max} and V/K_{Tyr} values at the pL optima gave ^{D_2O}V and $^{D_2O}V/K_{Tyr}$ values of 1.0 and 1.1, respectively. Neither is significantly different from 1, so that no solvent-exchangeable proton is transferred in the rate-limiting step. The effects of D_2O on the pK_a values are normal: the values from the V_{max} profile shift 6.0 ± 0.18 and 7.8 ± 0.12 in H_2O to 6.6 ± 0.25 and 8.2 ± 0.17 in D_2O, and the values from the V/K_{Tyr} profile shift from 6.6 ± 0.01 and 7.2 ± 0.01 in H_2O to 6.9 ± 0.01 and 7.5 ± 0.01 in D_2O.

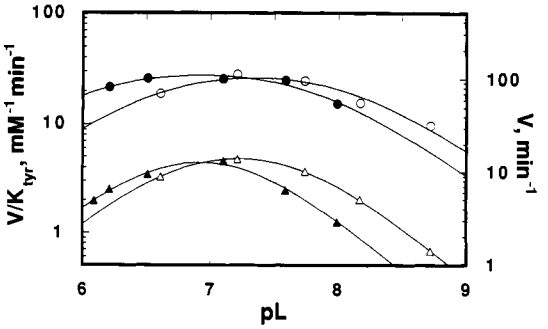


FIGURE 1: Solvent isotope effects on tyrosine hydroxylase. Initial rates of DOPA formation were determined at each pL value by varying the concentration of tyrosine in the presence of 550 μM 6-methyltetrahydropterin, 250 μM oxygen, 90 μg/mL catalase, 14 mM β-mercaptoethanol, 12 μM ferrous ammonium sulfate, 50 mM MES, and 50 mM Tris–acetate, at 30 °C. The data at each pL value were fit to $v = VS/(K_M + S)$ to give the indicated V_{max} and V/K_{Tyr} values. These values were then fit to log v = log $(C/(1 + [H^+]/K_a + K_b/[H^+]))$ to give the lines. (●, ○) V_{max} values in H_2O and D_2O, respectively; (▲, △) V/K_{Tyr} values in H_2O and D_2O, respectively.

Discussion

This report describes several probes of the identity of the rate-limiting step in the tyrosine hydroxylase catalyzed reaction. The lack of a burst of DOPA production with tyrosine as substrate has established that product release is rapid with this enzyme (Fitzpatrick, 1991). Consequently, the V_{max} value contains only the rates of chemical steps and conformational changes. The effects on the kinetic parameters of using substituted phenylalanines in place of tyrosine are summarized in Table I. The most surprising result is that the turnover number for the enzyme is essentially unaffected by the substituent on the aromatic ring of the amino acid. This is in contrast to the significant effect of meta substituents on the rate of oxygen transfer to the substrate by the flavoprotein phenol hydroxylase, where it is possible to follow the formation and decay of the flavin intermediates directly (Detmer & Massey, 1985).

Tyrosine is the last substrate to bind; therefore, the V/K_{Tyr} value will include the rate constants for all steps from binding through the first irreversible step. The V/K_{aa} value does not show any clear pattern other than a decreased affinity with increasing size at the para position of the amino acid substrate. The similar K_M values for 4-fluorophenylalanine and tyrosine and the much larger value for 4-methoxyphenylalanine show this especially clearly. We have found a similar trend with the bovine enzyme (Meyer, 1989).

Both of these trends are consistent with a lack of chemistry at the amino acid in the rate-limiting step of this enzyme. This is also consistent with the lack of a primary kinetic isotope effect on the reaction (Fitzpatrick, 1991). However, the lack of a primary kinetic isotope effect is consistent with any mechanism involving attack by an electrophilic oxygen species on the substrate. In contrast, if attack by an oxygenating species on the amino acid did occur in a slow step, the range of reactivities of the compounds in Table I would have resulted in a significant difference in the observed V_{max} values.

The possibility that the rate-limiting step is the dehydration of the 4a-hydroxytetrahydropterin to the quinonoid dihydropterin was considered, since that step can be limiting with phenylalanine hydroxylase (Lazarus, 1983). That possibility appears unlikely for several reasons: (1) Dehydration is typically found to be limiting under conditions of low concentrations of tetrahydropterin and high levels of phenylalanine hydroxylase, where the need for dehydration to the quinonoid dihydropterin before reduction by dihydropteridine reductase limits the amount of tetrahydropterin substrate available. The experiments described here were done under initial rate conditions where recycling was unnecessary. (2) The rate of dehydration of the hydroxytetrahydropterin is pH dependent. It is much more rapid at pH 6.5, the conditions of these experiments, than at pH 8 and above, the typical conditions for assays of phenylalanine hydroxylase (Shiman, 1985). (3) Detection of the hydroxypterin as a product of tyrosine hydroxylase is difficult, requiring enzyme concentrations some 20–50-fold higher than used in these studies, even to build up the species at pH 8 (Dix et al., 1987). This is due to the fact

that dehydration is still relatively rapid at pH 8. (4) Finally, the kinetic constants with tyrosine and 3-hydroxyphenylalanine reported in Table I were indistinguishable from values determined by measuring the rate of production of DOPA directly.

The substrate specificity of tyrosine hydroxylase has not been systematically examined. Several laboratories have tested phenylalanine as a substrate, with contradictory results. Using purified bovine enzyme, Shiman et al. (1971) and Haavik and Flatmark (1987) have reported that phenylalanine is a substrate for tyrosine hydroxylase, forming tyrosine as product. The former authors also reported that phenylalanine has a V_{max} value similar to that with tyrosine, consistent with the results in Table I. Tong et al. (1971), using a crude bovine adrenal preparation, reported that 3-hydroxyphenylalanine was also formed from phenylalanine. We have found that both tyrosine and 3-hydroxyphenylalanine are products when phenylalanine is used as substrate for tyrosine hydroxylase, with a ratio of tyrosine to 3-hydroxyphenylalanine produced of about 25.[2] In contrast, Dix et al. (1987) and Kuhn and Billingsley (1987), using the same preparation of rat tyrosine hydroxylase, were unable to detect any activity with phenylalanine as substrate, but did find that tryptophan is a substrate. The reasons for the discrepancy are unclear; however, it can be difficult to detect tyrosine production spectrophotometrically against the background of tetrahydropterin oxidation unless relatively high concentrations of enzyme (>0.2 μM) of high specific activity are used.

As a further probe of the identity of the rate-limiting step, solvent isotope effects were determined. No solvent isotope effect was detected on either the V_{max} or the V/K_{Tyr} value. Formation of hydroxylated amino acid products by tyrosine hydroxylase necessarily involves transfer of exchangeable protons. However, formation of a hydroxylating intermediate need not. The lack of an observed solvent kinetic isotope effect is readily accounted for if the rate-limiting step in the tyrosine hydroxylase reaction is formation of a peroxytetrahydropterin or other hydroxylating species. As noted above, the flavoprotein phenol hydroxylases provide a model system for pterin-dependent hydroxylases. The rate-limiting step in the reactions of both tetrahydropterins and reduced flavins with oxygen is abstraction of an electron from the cofactor to form the semiquinone and superoxide (Eberlein & Bruice, 1983; Eberlein et al., 1984). Subsequent recombination of the radical pair and proton transfer to form the peroxy cofactor are rapid. Thus, the rate-limiting step in model studies of the oxygen reaction involves no proton transfer. Also, the second-order rate constants for the reaction of molecular oxygen with the reduced flavin in *p*-hydroxybenzoate hydroxylase and phenol hydroxylase to form the peroxyflavin vary little with changes in the reactivity of the substrate or whether the substrate is even hydroxylated (Entsch et al., 1976; Detmer & Massey, 1985).

The mechanism of Scheme II is similar to previous proposals for hydroxylation of phenols by flavoprotein hydroxylases. Compounds III–V were tested as inhibitors, since they are structurally similar to the intermediates depicted in Scheme II. The lack of any inhibition by the phenoxide analogue III suggests that phenoxide formation is not obligatory for catalysis. Such a conclusion is confirmed by the ability of 4-methoxyphenylalanine to act as a substrate. With the flavoprotein hydroxylases, the un-ionized form of the phenolic substrate is bound preferentially, so that there is no direct evidence for involvement of the phenoxide in catalysis with those enzymes either (Schopfer & Massey, 1980; Detmer & Massey, 1985).

Compounds IV and V were tested as analogues of the proposed hydroxycyclohexadienone intermediate. The slight inhibition seen could be accounted for if these compounds bind as DOPA analogues, with a decrease in affinity due to replacement of the phenyl ring with a pyridyl ring. The weak inhibition is consistent with a lack of enzymatic catalysis of the formation of a hydroxycyclohexadienone intermediate in the hydroxylation of tyrosine by tyrosine hydroxylase. This result does not rule out the possibility that such a species forms. Since no uncoupled tetrahydropterin oxidation occurs in the absence of amino acid at pH 6.5, a conformational change must occur upon binding of the amino acid. It may be that IV and V are unable to trigger the conformational change to a form which binds preferentially to this intermediate. Alternatively, the enzyme may not need to actively catalyze the actual hydroxylation. Eberlein et al. (1984) have reported a limiting rate of reaction of oxygen and tetrahydropterin of 10^{-6} s^{-1}. The turnover number for tyrosine hydroxylase is about 2 s^{-1}, for a rate enhancement of about 2 million fold. Once formed, a 4a-peroxytetrahydropterin would be expected to be similar in reactivity to a 4a-peroxyflavin (Dix et al., 1985). The latter is a highly effective oxygen donor due to the electronegativity of the nitrogen substituents and the carbonyl group at the 4-position (Bruice, 1982), all of which tetrahydropterins share. It may only be necessary for tyrosine hydroxylase to bring together the amino acid and the peroxypterin in the active site for the reaction to occur sufficiently rapidly that other steps are rate-limiting. In that case, even if a hydroxycyclohexadienone is an intermediate in the hydroxylation reaction, the enzyme need not lower the activation energy for its formation.

The differences in the reactivities of the compounds tested as substrates can be calculated from their σ^+ values, if one neglects the steric effects for ortho substitution. The substrates range in reactivity from 4-aminophenylalanine to phenylalanine; the former compound has a value of −1.47 (Taylor, 1990). While the ρ values for electrophilic aromatic substitution vary over a wide range depending upon the specific reaction, even a relatively low value of −2 would predict almost a 1000-fold difference in reactivity. Thus, if one invokes a modified version of Scheme II in which phenolate formation is not required, hydroxylation of tyrosine must be far faster than formation of the hydroxylating intermediate to account for the results with phenylalanine.

Alternatively, the results can be explained by a mechanism involving attack of a hydroxyl radical on the substrate. In the case of tyrosine, this would form a hydroxycyclohexadienyl radical. Subsequent rapid transfer of a hydrogen atom would form the dihydroxylated product. Compounds IV and V would not be expected to be inhibitors if such a mechanism were operative. Such a mechanism has been proposed for the flavoprotein hydroxylases and can be used to explain the spectral changes that occur during the reaction catalyzed by *p*-hydroxybenzoate hydroxylase (Anderson et al., 1987). An analogous reaction at the active site of tyrosine hydroxylase would be homolytic cleavage of the tetrahydropterin hydroperoxide. Indeed, peroxides are known to homolytically cleave to form hydroxyl radicals in the presence of transition metals (Sheldon, 1983).

In conclusion, the results presented here establish that the rate-limiting step in the tyrosine hydroxylase reaction does not involve chemistry at the amino acid substrate and does not

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

involve general acid/base chemistry. All of the results presented are consistent with the primary role of the protein in tyrosine hydroxylase being catalysis of the formation of the hydroxylating intermediate in the vicinity of the bound amino acid substrate.

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