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Articles

3-Phenylpropenes as Mechanism-Based Inhibitors of Dopamine β-Hydroxylase: Evidence for a Radical Mechanism†

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ABSTRACT: A series of ring-substituted 3-phenylpropenes has been examined as mechanism-based inhibitors for the copper protein dopamine β-hydroxylase. *p*-HO-, *p*-CH_3O-, *m*-HO-, *m*-CH_3O-, *p*-Br-, and *p*-CN-substituted phenylpropenes all inactivate the enzyme under turnover conditions, requiring ascorbate and oxygen. Replacement of the benzylic hydrogens in 3-(*p*-hydroxyphenyl)propene with deuterium results in a kinetic isotope effect of 2.0 on k_{inact}/K_{O_2} but in no effect on the partition ratio, V_{max}/k_{inact}, consistent with a stepwise mechanism for hydrogen abstraction and oxygen insertion. The partition ratio is unchanged in the pH range from 4.5 to 7.1. Determination of the kinetics of inactivation and the partition ratios for each of these ring-substituted phenylpropenes has allowed determination of the respective V/K_{O_2} values. A linear free energy plot of these values as a function of σ^+ gives a ρ value of −1.2, while the partition ratios show only a slight decrease upon going from electron-donating to electron-withdrawing groups. The results are consistent with a mechanism for dopamine β-hydroxylase in which a hydrogen atom is abstracted to form a benzylic radical, which then partitions between hydroxylation and enzyme inactivation.

The physiological reaction catalyzed by the copper protein dopamine β-hydroxylase is the hydroxylation of dopamine to form norepinephrine, the final step in the biosynthesis of this important neurotransmitter. In vitro, however, the enzyme shows a broad substrate specificity (Creveling et al., 1962; Rosenberg & Lovenberg, 1980). It will hydroxylate benzyl cyanides, phenyl thioethers, phenethylamines, and phenylpropenes (Baldoni & Villafranca, 1980; May & Phillips, 1980; Klinman & Kreuger, 1982; Rajashekhar et al., 1984). This has been taken advantage of to probe the mechanism of C–H bond activation during catalysis, especially in the development of mechanism-based inhibitors or suicide substrates. Benzyl cyanides were the first class of compounds shown to inactivate

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dopamine β-hydroxylase under catalytic conditions, although the interpretation of the results in terms of an enzyme mechanism was complicated by the instability of the mandelonitrile product (Colombo et al., 1984a–c). β-Chlorophenethylamine also inactivates the enzyme during turnover, but the mechanism of inactivation is unknown (Klinman & Kreuger, 1982). We have recently shown that several 3-(*p*-hydroxyphenyl)propenes act as suicide substrates for dopamine β-hydroxylase (Rajashekhar et al., 1984; Colombo et al., 1984d), without the complications of these earlier inhibitors. While the mechanism initially proposed for inactivation by 2-bromo-3-(*p*-hydroxyphenyl)propene involved HBr loss to form an allene, this has been shown not to be the case (Rajashekhar et al., 1984). Instead, a carbanion or radical intermediate was proposed. This paper reports experiments that attempt to distinguish between these two possibilities and presents evidence for a radical intermediate in catalysis.

Experimental Procedures

Materials. Ascorbic acid, disodium fumarate, and 2-(*N*-morpholino)ethanesulfonic acid (MES)[1] were from Sigma Chemical Co. Acetic acid, DMF, tyramine hydrochloride, vinylmagnesium bromide, *p*-methoxybenzaldehyde, *m*-methoxybenzaldehyde, *p*-cyanobenzaldehyde, *p*-bromobenzaldehyde, boron tribromide, 3-phenylpropene, *p*-fluorophenylmagnesium bromide, lithium aluminum hydride, 1,4-dibromobenzene, allyl bromide, allyl iodide, and thionyl chloride were from Aldrich. 3-(*p*-Methylphenyl)propene was from K & K. Cuprous cyanide and silica (40–140 mesh) were from Baker. Methyl anisate was from Kodak. Lithium aluminum deuteride was from Merck Sharp & Dohme. Magnesium turnings were from Alfa. Catalase was from Boehringer-Mannheim.

Methods. Dopamine β-hydroxylase was purified and assayed as previously described (Rajashekhar et al., 1984). All kinetic data were fit by computer to the hyperbolic form of the relevant rate equation by using the programs of Cleland (1979) as modified by Dr. David Clark of this laboratory. For determination of inactivation kinetics, a typical reaction mixture contained 36 mM ascorbate, 0.2–0.4 mg/mL catalase, 14% DMF, 15–30 μg of dopamine β-hydroxylase, and 0.1 M MES–0.1 M sodium acetate, pH 5.5, in 0.25 mL at 25 °C. The reaction mixture was stirred for 5–10 min at 25 °C under the appropriate concentration of oxygen before adding either ascorbate or enzyme through a rubber septum to initiate the reaction. Aliquots were withdrawn at 3–7-min intervals through the septum and assayed with tyramine as substrate at 25 °C. A similar reaction mixture was used for determining partition ratios, except that the volume was 0.1 mL, and all reactions were done at 1.21 mM oxygen. At 5–10-min intervals 20-μL aliquots were withdrawn and mixed with 40 μL of tetrahydrofuran/methanol (2:1) on ice. The amount of product was determined by HPLC using a Waters HPLC system with a reverse-phase C-18 column as described previously (Rajashekhar et al., 1984). The column was eluted with acetonitrile/50 mM acetic acid; the amount of each component varied, depending upon the inhibitor being analyzed, from 70/30 for 3-(*p*-hydroxyphenyl)propene and 3-(*m*-hydroxyphenyl)propene to 50/50 for 3-(*p*-bromophenyl)propene. The retention times and extinction coefficients at 280 nm were determined for most of the compounds by using authentic synthetic products. For 3-(*p*-hydroxyphenyl)propene and 3-(*m*-hydroxyphenyl)propene, the products were synthesized enzymatically as described previously (Rajashekhar et al., 1984). The product with 3-(*p*-bromophenyl)propene was detected at 254 nm. For those inhibitors that had $t_{1/2}$'s of less than 5 min under these conditions, the amount of product at complete inactivation was determined. For the remainder the time course of product formation was fit to a logarithmic curve to determine the end point. In all cases the rate of product formation was the same as the rate of enzyme inactivation under the same conditions, as expected (Waley, 1980). For calculations of partition ratios it was assumed that there are four active sites per tetramer of M_r 290 000, since it has been shown that dopamine β-hydroxylase contains eight copper atoms per tetramer (Ash et al., 1984; Klinman et al., 1984). Control experiments showed that the partition ratios determined in this manner were independent of the concentrations of oxygen and enzyme.

Syntheses. 3-(*p*-Hydroxyphenyl)propene was synthesized as previously described (Rajashekhar et al., 1984). *p*-Methoxy[*methylene*-$^2H_2$]benzyl chloride was synthesized by the method of Monti et al. (1979). Forty milliliters of 1.0 M vinylmagnesium bromide in tetrahydrofuran was added dropwise to 4.5 g of *p*-methoxy[*methylene*-$^2H_2$]benzyl chloride with stirring in 40 mL of dry tetrahydrofuran under N_2 at room temperature. After the solution was refluxed for 20 h, the reaction mixture was poured into 100 mL of ice-cold 10% HCl. This was extracted twice with 100 mL of diethyl ether, and the organic layers were pooled and dried over magnesium sulfate. The solvent was then removed under vacuum. This was purified by silica gel chromatography in petroleum ether/benzene (4:1) followed by vacuum distillation to give 1.27 g of 3-(*p*-methoxyphenyl)[3,3-$^2H_2$]propene: ^1H NMR δ 3.8 (3 H, s), 4.8–5.3 (2 H, m), 5.7–6.3 (1 H, m), 6.8–7.3 (4 H, dd, J = 8, 20 Hz); mass spectrum, m/e 150. This was converted to 3-(*p*-hydroxyphenyl)[3,3-$^2H_2$]propene with 2 equiv of BBr_3 as previously described for 3-(*p*-hydroxyphenyl)-propene (Rajashekhar et al., 1984): ^1H NMR δ 4.8–5.2 (2 H, m), 5.3 (1 H, s, exchangeable), 5.7–6.3 (1 H, m), 6.7–7.2 (4 H, dd, J = 8, 20 Hz); mass spectrum, m/e 136.

For 3-(*m*-methoxyphenyl)propene, 1.44 g of Mg was stirred in 50 mL of dry tetrahydrofuran at room temperature under nitrogen. One gram of *m*-bromoanisole was added dropwise followed by 0.1 mL of dibromoethane. After the reaction mixture began to warm, the remainder of 5 g of *m*-bromoanisole was added at a rate sufficient to keep the reflux controlled. Thirty minutes after the final addition was complete, the reaction mixture was filtered into an addition funnel and added dropwise to 4.81 g of allyl iodide in 50 mL of dry ether under nitrogen. The reaction was stirred overnight. It was then poured into 150 mL of crushed ice containing 25 mL of HCl. This was extracted twice with ether, the organic layers were pooled and dried over magnesium sulfate, and the solvent was removed under vacuum. Purification by silica chromatography in hexane/benzene (3:1) followed by vacuum distillation gave 1.04 g of 3-(*m*-methoxyphenyl)propene: ^1H NMR δ 3.2–3.4 (2 H, d, J = 6 Hz), 3.8 (3 H, s), 4.9–5.3 (2 H, m), 5.7–6.4 (1 H, m), 6.7–7.4 (4 H, m); mass spectrum, m/e 148.

This was demethylated with BBr_3 by the method of McOmie et al. (1968). Ten milliliters of 1.0 M BBr_3 in methylene chloride was added to 0.73 g of 3-(*m*-methoxyphenyl)propene in 100 mL of dry methylene chloride at room temperature under nitrogen. After being stirred for 90 min, the reaction mixture was poured into 100 mL of 3.2% Na_2CO_3. This was

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; DMF, *N*,*N*-dimethylformamide; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; SD, standard deviation.

extracted 3 times with methylene chloride, and the pooled organic layers were extracted with water. They were then dried over magnesium sulfate, and the solvent was removed under vacuum. Purification by silica chromatography in chloroform followed by vacuum distillation gave 0.24 g of 3-(*m*-hydroxyphenyl)propene: ^{1}H NMR δ 3.2–3.4 (2 H, d, J = 6 Hz), 4.9–5.2 (3 H, m), 5.5–6.3 (1 H, m), 6.5–7.3 (4 H, m); mass spectrum, m/e 134.

3-(*p*-Bromophenyl)propene was synthesized by the method of Bilow et al. (1967). 3-(*p*-Chlorophenyl)propene and 3-(*p*-fluorophenyl)propene were synthesized by the procedure of Martin & Gleicher (1963). The synthesis of 3-(*p*-cyanophenyl)propene was based on the method of Friedman & Schechter (1961). 3-(*p*-Bromophenyl)propene (1.9 g) and 2 g of cuprous cyanide were refluxed for 5 h in 2.5 mL of DMF. The reaction mixture was then poured into 7 mL of 17% HCl containing 4 g of $FeCl_3 \cdot 6H_2O$ and stirred for 20 min at 60–70 °C. This was extracted twice with 10 mL of toluene. The pooled organic layers were washed with 2.5 mL of 50% HCl followed by 3.5 mL of 2 M NaOH, filtered, and dried over magnesium sulfate before removing the solvent under vacuum. The crude product was purified by distillation at 80–81 °C at 3–4 mm [lit. mp 128–129 °C, 18 mm (Camaggi et al., 1977)] to give 0.45 g of 3-(*p*-cyanophenyl)propene.

To synthesize 3-hydroxy-3-(*m*-methoxyphenyl)propene, 25 mL of 1.0 M vinylmagnesium bromide in tetrahydrofuran was added dropwise to 3.4 g of *m*-methoxybenzaldehyde with stirring in 60 mL of dry ether at −78 °C under N_2. After 4 h 25 mL of 10% NH_4Cl was added slowly and the reaction mixture warmed to room temperature. This was extracted 4 times with 75 mL of ether. After the reaction mixture was dried over sodium sulfate, the solvent was removed under vacuum. Purification by silica chromatography in $CHCl_3$/ethyl acetate (19:1) yielded 1.17 g of 3-hydroxy-3-(*p*-methoxyphenyl)propene: ^{1}H NMR δ 2.4 (1 H, s, exchangeable), 3.8 (3 H, s), 5.0–5.3 (2 H, m), 5.4 (1 H, s), 5.7–6.3 (1 H, m), 6.7–7.4 (4 H, m); mass spectrum, m/e 164.

To synthesize 3-hydroxy-3-(*p*-methoxyphenyl)propene, 25 mL of 1.0 M vinylmagnesium bromide in tetrahydrofuran was added dropwise to 3.4 g of *p*-methoxybenzaldehyde in 100 mL of tetrahydrofuran with stirring under nitrogen at −35 °C. After 2 h the reaction mixture was poured into 50 mL of 10% NH_4Cl. This was extracted with ether and dried over magnesium sulfate. Distillation and silica chromatography as for 3-hydroxy-3-(*m*-methoxyphenyl)propene gave 0.7 g of 3-hydroxy-3-(*p*-methoxyphenyl)propene: ^{1}H NMR δ 2.4 (1 H, s, exchangeable), 3.8 (3 H, s), 5.1–5.3 (2 H, m), 5.5 (1 H, m), 5.8–6.4 (1 H, m), 6.8–7.5 (4 H, dd, J = 8, 25 Hz); mass spectrum, m/e 164.

For 3-hydroxy-3-(*p*-cyanophenyl)propene, 14 mL of 1.0 M vinylmagnesium bromide in tetrahydrofuran was added dropwise to 1.82 g of *p*-cyanobenzaldehyde in 50 mL of dry tetrahydrofuran with stirring under nitrogen at −35 °C. After being stirred for 3 h, this was poured into 50 mL of 10% NH_4Cl. The reaction mixture was extracted with ether and dried over magnesium sulfate; the ether was removed under vacuum. Purification by silica chromatography in chloroform and vacuum distillation gave 0.74 g of 3-hydroxy-3-(*p*-cyanomethyl)-propene: ^{1}H NMR δ 2.5 (1 H, s), 5.1–5.3 (2 H, m), 5.4–5.5 (1 H, m), 5.7–6.4 (1 H, m), 7.3–7.7 (4 H, dd, J = 9, 12 Hz); mass spectrum, m/e 159.

3-Hydroxy-3-(*p*-bromophenyl)propene was synthesized in a similar fashion by adding 25 mL of 1.0 M vinylmagnesium bromide to 4.62 g of *p*-bromobenzaldehyde with stirring under nitrogen in dry tetrahydrofuran at −35 °C. After being stirred for 3 h, the reaction mixture was poured into 50 mL of 10% NH_4Cl. Workup as above followed by vacuum distillation (80 °C, 1-2 mm) gave 3.05 g of 3-hydroxy-3-(*p*-bromophenyl)-propene: ^{1}H NMR δ 2.7–2.9 (1 H, s), 4.9–5.4 (3 H, m), 5.6–6.3 (1 H, m), 7.0–7.5 (4 H, dd, J = 8, 17 Hz); mass spectrum, m/e 212, 214.

Table I: Kinetics of Inactivation of Dopamine β-Hydroxylase by 3-Phenylpropenes[a]

inhibitor	k_{inact} (min^{-1})[b]	K_I (mM)[b]
3-phenylpropene	0.048	3.6
3-(*p*-fluorophenyl)propene	0.083	3.6
3-(*p*-bromophenyl)propene	0.021	0.34
3-(*p*-chlorophenyl)propene	0.025	1.4
3-(*p*-methylphenyl)propene	0.025	1.6
3-(*p*-hydroxyphenyl)propene	0.51	1.8
3-(*p*-methoxyphenyl)propene	0.13	4.0
3-(*p*-cyanophenyl)propene	0.012	1.5
3-(*m*-hydroxyphenyl)propene	0.14	12
3-(*m*-methoxyphenyl)propene	0.019	7.6

[a] Conditions: 25 °C, 14% DMF, 0.254 mM oxygen, 36 mM ascorbate, 0.2 mg/mL catalase, 0.1 M MES, 0.1 M sodium acetate, pH 5.5. Dopamine β-hydroxylase (1–3 μM) was preincubated with varying concentrations of the indicated inhibitor, and the rate of loss of activity was determined by withdrawing aliquots and measuring the residual activity with tyramine as substrate. [b] The observed rates of inactivation were fit to the hyberbolic form of the rate equation $1/k_{obsd} = 1/k_{inact} + K_I/k_{inact}[I]$. Thus, K_I is an apparent binding constant and is not meant to represent either a K_d or a true K_m.

Results

In order to obtain information as to the structure of catalytic intermediates during inactivation of dopamine β-hydroxylase by 3-phenylpropenes, the effect of substitution in the benzene ring was examined. *p*-HO-, *p*-CH_3O-, *p*-F-, *p*-H-, *p*-Cl-, *p*-Br-, *p*-CH_3-, *p*-CN-, *m*-HO-, and *m*-CH_3O-substituted phenylpropenes were all found to inactivate the enzyme under turnover conditions. All fit the criteria for mechanism-based inhibitors (Abeles & Maycock, 1976): the inactivation required the presence of the cosubstrates oxygen and ascorbate, the inactivation was prevented by the presence of the substrate tyramine, and the inactivation was first order and showed saturation kinetics. The kinetic parameters for inactivation in air-saturated buffer are listed in Table I. 3-(*p*-Hydroxyphenyl)propane (1 mM) did not inactivate the enzyme, indicating that the vinylic moiety was necessary. Interestingly, the presence of the anion activator fumarate (10 mM) had no effect upon the inactivation by phenylpropenes. Also, the presence of 50 mM mannitol as a free radical trap had no effect on the rates of inactivation.

Several of these phenylpropenes were chosen for a more complete analysis. As a rule, the ones chosen were the more water-soluble compounds, although it was possible to characterize inactivation by 3-(*p*-bromophenyl)propene because of its relatively low K_m value. The effects of substitution upon two parameters of inactivation were examined: the partition ratio (V_{max}/k_{inact}) and the kinetics of inactivation. The results of a typical kinetic analysis are shown in Figure 1A for 3-(*p*-hydroxyphenyl)propene. The concentration of ascorbate used was saturating, so it was not varied. A pattern of intersecting lines, consistent with eq 1, was seen, as was the case

$$1/k_{obsd} = 1/k_{inact} + K_I/k_{inact}[I] + K_{O_2}/k_{inact}[O_2] + K_{iI}K_{O_2}/k_{inact}[I][O_2] \quad (1)$$

with all of the inhibitors tested. Replacement of the benzylic hydrogens with deuterium resulted in a kinetic isotope effect of 2.0 on k_{inact}/K_{O_2} (Figure 1B). Because the K_m for oxygen

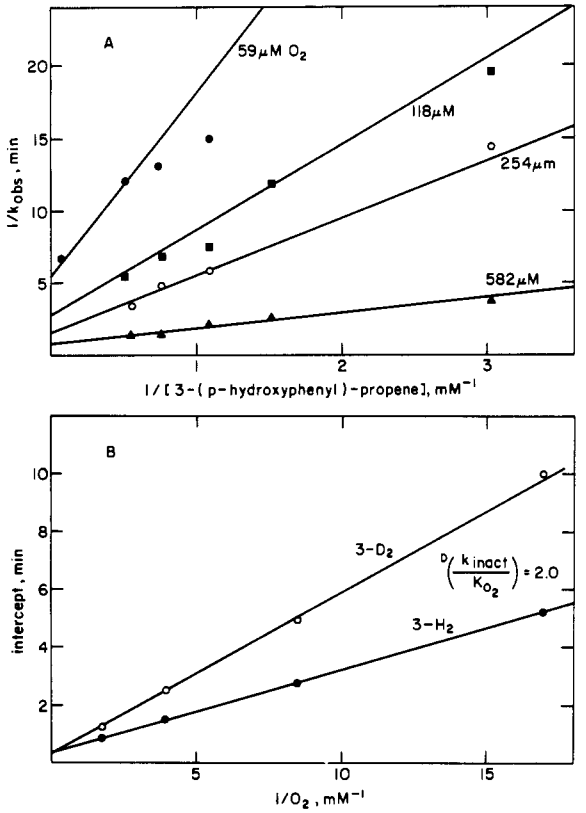

FIGURE 1: (A) Double-reciprocal plot of inactivation of dopamine β-hydroxylase by 3-(*p*-hydroxyphenyl)propene. Conditions: 36 mM ascorbate, 14% DMF, 0.2 mg/mL catalase, 0.1 M MES, 0.1 M sodium acetate, pH 5.5, at 25 °C. Enzyme (1–2 μM) was preincubated with the indicated concentrations of inhibitor and oxygen. Aliquots were removed at various times, and residual activity was determined as described under Experimental Procedures. The data were computer fit to $k_{obsd} = k_{inact}[I][O_2]/([I][O_2] + K_I[O_2] + [I]K_{O_2} + K_{II}K_{O_2})$. (B) Kinetic isotope effect on k_{inact}/K_{O_2}. The intercepts from (A) and from a similar experiment with 3-(*p*-hydroxyphenyl)[3,3-$^2H_2$]propene are shown as a function of oxygen concentration.

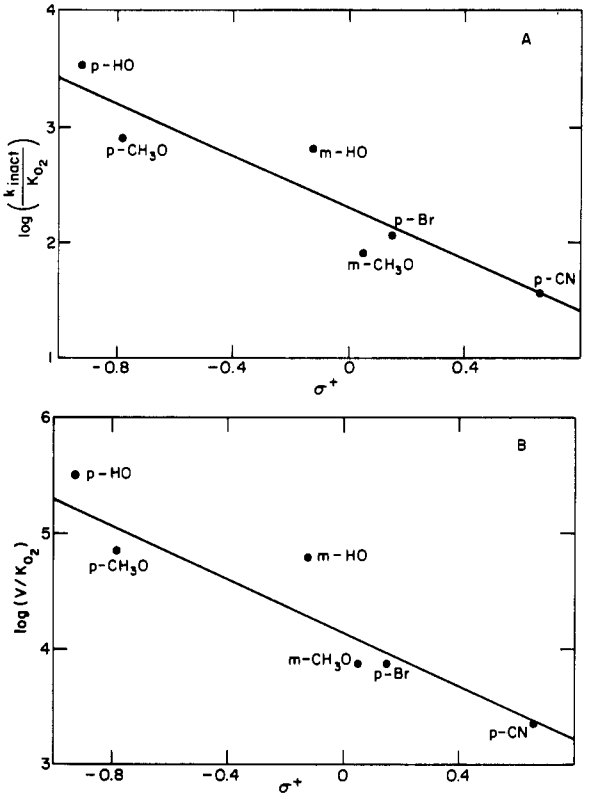

FIGURE 2: (A) Linear free energy plot of k_{inact}/K_{O_2} values for 3-phenylpropenes as mechanism-based inhibitors of dopamine β-hydroxylase. Dopamine β-hydroxylase was incubated with varying amounts of the indicated inhibitors and 59 μM to 1.21 mM oxygen in 36 mM ascorbate, 0.2–0.4 mg/mL catalase, 14% DMF, 0.1 M MES, and 0.1 M sodium acetate, pH 5.5 at 25 °C. (B) Linear free energy plot of V/K_{O_2} values for 3-phenylpropenes as substrates for dopamine β-hydroxylase. The V/K_{O_2} values were calculated by multiplying k_{inact}/K_{O_2} values by the respective partition ratios. The σ^+ values are from Ritchie & Sager (1964).

(0.9 mM) was higher than the highest oxygen concentration at which we could accurately measure rates of inactivation (0.6 mM), we were unable to determine accurately the isotope effects upon the other kinetic parameters. Similar analyses were done for several ring-substituted 3-phenylpropenes. A linear free energy plot of the values of k_{inact}/K_{O_2} determined in this fashion as a function of the electron-donating or -withdrawing ability of the ring substituent is shown in Figure 2A. A good correlation was obtained with σ^+, giving a ρ value of −1.2.

The partition ratio for phenylpropenes was determined by measuring the amount of product present after complete inactivation of dopamine β-hydroxylase. The product of hydroxylation of 3-(*p*-hydroxyphenyl)propene has been shown to be 3-hydroxy-3-(*p*-hydroxyphenyl)propene by mass spectral analysis (Rajashekhar et al., 1984). For *p*-Br-, *p*-CH_3O-, *m*-CH_3O-, and *p*-CN-substituted phenylpropenes, the enzymatic products had identical retention times on an HPLC column with authentic 3-hydroxy-3-(*p*-X-phenyl)propenes, indicating that all of the phenylpropenes analyzed are hydroxylated at the benzylic position. As shown in Table II, the partition ratio for 3-(*p*-hydroxyphenyl)propene changes very little between pH 4.5 and pH 7.1, although the observed rate of inactivation was about 10-fold lower at pH 7.1 than at pH 5.5. Also, replacement of the benzylic hydrogens with deuterium has no significant effect on the partition ratio (Table II). The partition ratios for several other phenylpropenes were also determined and these results are shown in Table III.

Table II: Partition Ratios for 3-(*p*-Hydroxyphenyl)propene as a Mechanism-Based Inhibitor of Dopamine β-Hydroxylase

inhibitor	pH	partition ratio (SD)
Experiment 1		
3-(*p*-hydroxyphenyl)propene	4.5	76 (5)
3-(*p*-hydroxyphenyl)propene	5.5	84 (9)
3-(*p*-hydroxyphenyl)propene	6.5	94 (5)
3-(*p*-hydroxyphenyl)propene	7.1	79 (14)
Experiment 2		
3-(*p*-hydroxyphenyl)[3,3-$^1H_2$]propene	5.5	96 (11)
3-(*p*-hydroxyphenyl)[3,3-$^2H_2$]propene	5.5	91 (6)

[a] Dopamine β-hydroxylase (3–5 μM) was preincubated at 25 °C with 4.7 mM 3-(*p*-hydroxyphenyl)propene, 0.4 mg/mL catalase, 36 mM ascorbate, 1.21 mM oxygen, 14% DMF, 0.1 M MES, and 0.1 M sodium ascorbate, at the indicated pH. After complete inactivation of the enzyme, the amount of product present was determined as described under Experimental Procedures.

Discussion

All of the phenylpropenes discussed here fulfill the criteria for mechanism-based inhibitors of dopamine β-hydroxylase. These compounds only inactivate under turnover conditions, they are also substrates for the enzyme, inactivation is prevented by the substrate tyramine, and the kinetics of inactivation are first order and exhibit saturation behavior.

2-Bromo-3-(*p*-hydroxyphenyl)propene, the first of the phenylpropene series to be reported as a suicide substrate for dopamine β-hydroxylase, was initially proposed to inactivate the enzyme through loss of HBr to form a highly reactive

Table III: Partition Ratios for Inactivation of Dopamine β-Hydroxylase by Phenylpropenes[a]

inhibitor	partition ratio (SD)
3-(*p*-hydroxyphenyl)propene	96 (11)
3-(*p*-methoxyphenyl)propene	87 (5)
3-(*m*-hydroxyphenyl)propene	107 (6)
3-(*m*-methoxyphenyl)propene	99 (7)
3-(*p*-bromophenyl)propene	64 (24)
3-(*p*-cyanophenyl)propene	63 (4)

[a] Conditions: 36 mM ascorbate, 1.21 mM oxygen, 1–5 mM phenylpropene, 3–5 μM dopamine β-hydroxylase, 14% DMF, 0.1 M MES, 0.1 M sodium acetate, pH 5.5, 25 °C. The partition ratios were determined as described under Experimental Procedures.

allene (Colombo et al., 1984d). This is a mechanism for which there is ample precedent for enzymes which stabilize carbanions [for example, see Abeles & Maycock (1976)]. However, the lack of an effect upon the partition ratio of replacement of the Br with H ruled out this possibility. The necessity of the unsaturated propene moiety for inactivation suggested that the catalytic intermediate might be a resonance-stabilized radical or carbanion (Rajashekhar et al., 1984). The experiments described in this paper were designed to examine the effect of varying the substituents on the benzene ring upon inactivation in order to distinguish between these two possibilities.[2]

In order to analyze the effect of substituents upon inactivation, we measured two parameters associated with inactivation, the kinetics of inactivation and the partition ratio. Miller & Klinman (1983) have shown that eq 2 applies for

$$1/v = 1/V + K_A/V[A] + K_B/V[B] + K_{O_2}/V[O_2] + K_{iB}K_{O_2}/V[B][O_2] \quad (2)$$

the steady-state kinetics of dopamine β-hydroxylase with dopamine as substrate, where A is ascorbate and B is dopamine. The simplest kinetic mechanism consistent with this is eq 3.

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \xrightarrow{k_3} E' \underset{k_6}{\overset{k_5 B}{\rightleftharpoons}} E'B \underset{k_8}{\overset{k_7 O_2}{\rightleftharpoons}} E'BO_2 \xrightarrow{k_9} EP \xrightarrow{k_{11}} E \quad (3)$$

For such a mechanism, $V/K_{O_2} = k_7k_9/(k_8 + k_9)$ and is the kinetic parameter which contains k_9, the rate constant for the catalytic steps.[3] That k_{inact}/K_{O_2} does indeed contain the catalytic step is shown by the isotope effect upon this parameter when the benzylic hydrogens are replaced by deuterium. The value of 2.0 found here is less than the observed value of 4–5 found when dopamine is the substrate (Ahn & Klinman, 1983) and much less than the value of 9.4 for the intrinsic isotope effect when dopamine is the substrate (Miller & Klinman, 1983). While Ahn & Klinman (1983) have shown that the actual kinetic mechanism is much more complicated

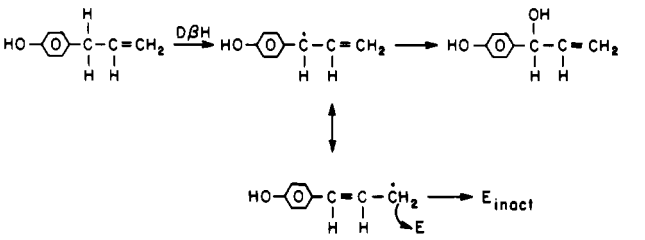

FIGURE 3: Proposed mechanism for mechanism-based inactivation of dopamine β-hydroxylase by 3-phenylpropenes.

than eq 3 due to dissociation of substrate from the ternary complex, the value of V/K_{O_2} remains $k_7k_9/(k_8 + k_9)$ despite these complications. As a more practical matter, V/K_{O_2} can be accurately determined even when it is not possible to measure V_{max} accurately either because of solubility problems or because it is too rapid. We have thus chosen to examine the effect of ring substitution upon this parameter.

Equation 1 is identical in form with eq 2 at infinite ascorbate. Indeed, because the partition ratio for inactivation by a suicide substrate is V_{max}/k_{inact}, inactivation kinetic data can be converted to steady-state kinetic data simply by multiplying by the partition ratio. That such an analysis is valid for this system is shown by the excellent agreement between partition ratios determined by measuring the amount of product produced after complete inactivation by 2-substituted 3-(*p*-hydroxyphenyl)propenes and that calculated from V_{max} and k_{inact} values (Rajashekhar et al., 1984). We have thus calculated values for V/K_{O_2} from the k_{inact}/K_{O_2} values by multiplying the latter by the corresponding partition ratios.[4] A linear free energy plot of V/K_{O_2} values determined in this fashion is shown in Figure 2B. A good correlation was obtained with σ^+, with a ρ value of −1.2. Use of σ, σ^n, σ^o, or σ^- gave a worse correlation.

With D-amino acid oxidase, a flavoprotein which goes through a carbanion intermediate (Ghisla, 1982), a ρ value of 5.44 was obtained when V_{max} was measured for a series of substituted phenylglycines (Neims et al., 1966). Thus, a carbanion mechanism can be ruled out for dopamine β-hydroxylase. Instead, a radical mechanism is consistent with both the small absolute magnitude of the ρ value and the correlation with σ^+ (Howard & Ingold, 1963; Kennedy & Ingold, 1966). Abstraction of a benzylic hydrogen from a series of phenylpropenes by the $CCl_3\cdot$ radical shows a ρ value of −0.6, while hydrogen abstraction by Br· gives a ρ value of −0.8 (Martin & Gleicher, 1963). Benzylic hydrogen abstraction from a series of substituted toluenes correlates well with σ^+ values, with the value of ρ varying from −0.4 to −1.8, depending upon the electron affinity of the attacking radical (Howard & Ingold, 1963). In contrast, a ρ value of 0.6 has been found for hydrogen abstraction from substituted toluenes by the *tert*-butyl radical; this was attributed to the nucleophilic character of that radical (Dutsch & Fischer, 1982). Thus, whether a ρ value for a radical reaction is positive or negative can be

[2] There is a previous report of the effect of such substitutions upon the steady-state kinetics of dopamine β-hydroxylase (May et al., 1981). However, these authors looked at the effect upon the apparent V_{max} values at a single oxygen concentration, ignoring the differences in K_m values for the various compounds. Also, because product release is the major factor in V_{max} (Miller & Klinman, 1983), in measuring changes in V_{max}, one is effectively measuring changes in the rate of product release rather than in the rate of chemical steps. Thus, while May et al. (1981) found a very poor correlation with σ^n over a very narrow range of values, the meaning of this result is ambiguous. This is further complicated by the fact that these authors corrected several of their numbers rather arbitrarily for steric effects.

[3] This analysis assumes that there is no consistent effect of electron donation on k_7/k_8. Since dopamine can dissociate from the ternary complex after oxygen binding (Ahn & Klinman, 1983), is it unlikely that there are strong interactions between oxygen and the hydroxylated substrate in the initial binding of oxygen.

[4] A more complete kinetic scheme accounting for both catalysis and inactivation from a common intermediate in the absence of products is

$$E \underset{k_2}{\overset{k_1, A}{\rightleftharpoons}} EA \xrightarrow{k_3} E' \underset{k_6}{\overset{k_5, I}{\rightleftharpoons}} E'I \underset{k_8}{\overset{k_7, O_2}{\rightleftharpoons}} E'IO_2 \xrightarrow{k_9} EX \xrightarrow{k_{13}} EP$$
$$EX \xrightarrow{k_{15}} E_{inact} \qquad EP \xrightarrow{k_{11}} E$$

For such a mechanism V/K_{O_2} remains $k_7k_9/(k_8 + k_9)$, and the partition ratio is k_{13}/k_{15}. Such a mechanism is kinetically indistinguishable from eq 3 for initial rate data. Miller & Klinman (1983) were unable to detect any exchange of benzylic hydrogens with solvent; thus, k_9 is assumed to be irreversible.

determined by whether the abstracting species is electrophilic or nucleophilic, with the magnitude of the value depending on the strength of the electrophile. A mechanism which is consistent with the ρ value of −1.2 found in this study would be abstraction of a hydrogen atom from the benzylic position of the phenylpropene substrate by an electrophilic species to form the benzylic radical. Resonance stabilization of the radical by the propene side chain would result in significant radical character at carbon 1. If an appropriate enzyme group were near to C-1, attack could result in inactivation of the enzyme (Figure 3).

The lack of an isotope effect upon the partition ratio for 3-(*p*-hydroxyphenyl)propene indicates that the C–H bond has already been broken in the catalytic intermediate which partitions between catalysis and inactivation. This indicates that C–H bond cleavage and oxygen insertion do not occur in a concerted fashion during catalysis. Such a result also rules out a mechanism in which a proton is abstracted from the product to form a resonance-stabilized carbanion which could inactivate the enzyme.[5]

There is only a very slight decrease in the partition ratio upon going from strongly electron-donating to strongly electron-withdrawing groups, especially if only the para-substituted compounds are examined (Table III). This is further evidence that hydrogen abstraction and inactivation are not concerted, since there is a large effect on hydrogen abstraction (Figure 2). A possible explanation for the observed decrease is that there is an increase in the reactivity of the radical with increased electron withdrawal. An increase in reactivity would lead to a decrease in the selectivity of the radical, so that it would be more likely to react with a group on the protein rather than the copper–oxygen species. The higher partition ratios with the meta-substituted compounds may be due to steric effects.

Studies of the effect of pH upon inactivation by 2-bromo-3-(*p*-hydroxyphenyl)propene identified a group with a pK of 5.7 as being involved in inactivation (Colombo et al., 1984d). Also, Ahn & Klinman (1983) identified a group with a pK of 5.6 in a study of the effect of pH upon the kinetics with dopamine as substrate. The lack of a pH effect upon the partition ratio for 3-(*p*-hydroxyphenyl)propene (Table II) indicates that the protonation state of this group is important for catalysis but not for inactivation. Further, the effect of pH upon the partition ratio for inactivation of dopamine β-hydroxylase by *m*-hydroxybenzyl cyanide is probably due to a pH effect upon the stability of the product mandelonitrile or the enzyme–cyanide complex (Colombo et al., 1983a).

The identity of the electrophilic species involved in hydrogen atom abstraction cannot be determined from the results of this study. A reasonable possibility is a high potential copper–oxygen species. Model studies have led to the proposal of a (μ-oxo)dicopper(III) species in the hydroxylation of stilbene (Franklin et al., 1984). Several features of dopamine β-hydroxylase are similar to those of cytochrome P-450-catalyzed hydroxylation of aliphatic carbons for which the proposed mechanism involves hydrogen atom abstraction by a high potential oxyferryl species (Groves et al., 1978). These include large intrinsic isotope effects (Miller & Klinman, 1983; Groves et al., 1978; Miwa et al., 1984) and inactivation by vinylic compounds (this study; Ortiz de Montellano et al., 1982). It has recently been shown that the allylic radical formed during hydroxylation of cyclohexene by cytochrome P-450 is long-lived enough that allylic rearrangement can occur (Groves & Subramanian, 1984). This is very similar to the mechanism of inactivation proposed above for dopamine β-hydroxylase.

[5] A model for inactivation by proton removal from enzyme-bound product to give the carbanion would be consistent with the lack of an observed isotope effect if the proton removal were extremely rapid compared to subsequent steps. The simplest kinetic mechanism for this is

$$E + PH \xrightarrow{k_5} EPH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EP^- \xrightarrow{k_3} E_{inact}$$

where PH is protonated product. For no isotope effect to be seen, $k_1 \gg k_2, k_3, k_5$. Under these conditions, $k_{inact} = k_3$ and $V_{max} = k_2k_5/(k_1 + k_5)$. If one assumes an intrinsic isotope effect for removal of the benzylic proton from product of 8 on k_1, such a model will fit the data for 3-(*p*-hydroxyphenyl)propene if $k_1 = 5 \times 10^5$ min^{-1} and $k_2 = 10^4$ min^{-1}, giving $k_5 = 920$ min^{-1} and an observed isotope effect upon the partition ratio of 1.25. Larger values of k_1 will of course give smaller isotope effects. This means that the enzyme must be capable of lowering the pK of the benzylic proton of the product to 3.8, since these values were determined at pH 5.5. Further, this proton must not exchange with solvent, since Miller & Klinman (1983) have shown that no exchange of benzylic protons occurs with dopamine. Finally, one must keep in mind that the proton involved is not the one which is removed during the normal course of catalysis. For these reasons, it is extremely unlikely that such a mechanism is correct.

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Secondary Isotope Effects and Structure–Reactivity Correlations in the Dopamine β-Monooxygenase Reaction: Evidence for a Chemical Mechanism†

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ABSTRACT: The chemical mechanism of hydroxylation, catalyzed by dopamine β-monooxygenase, has been explored with a combination of secondary kinetic isotope effects and structure–reactivity correlations. Measurement of primary and secondary isotope effects on V_{max}/K_m under conditions where the intrinsic primary hydrogen isotope effect is known allows calculation of the corresponding intrinsic secondary isotope effect. By this method we have obtained an α-deuterium isotope effect, $^{D}k_{\alpha} = 1.19 \pm 0.06$, with dopamine as substrate. The β-deuterium isotope effect is indistinguishable from one. The large magnitude of $^{D}k_{\alpha}$, together with our previous determination of a near maximal primary deuterium isotope effect of 9.4–11, clearly indicates the occurrence of a stepwise process for C–H bond cleavage and C–O bond formation and hence the presence of a substrate-derived intermediate. To probe the nature of this intermediate, a structure–reactivity study was performed by using a series of para-substituted phenylethylamines. Deuterium isotope effects on V_{max} and V_{max}/K_m parameters were determined for all of the substrates, allowing calculation of the rate constants for C–H bond cleavage and product dissociation and dissociation constants for amine and O_2 loss from the enzyme–substrate ternary complex. Multiple regression analysis yielded an electronic effect of $\rho = -1.5$ for the C–H bond cleavage step, eliminating the possibility of a carbanion intermediate. A negative ρ value is consistent with formation of either a radical or a carbocation; however, a significantly better correlation is obtained with σ_p rather than σ_p^+, implying formation of a radical intermediate via a polarized transition state. Additional effects determined from the regression analyses include steric effects on rate constants for substrate hydroxylation and product release and on $K_{D\,amine}$, consistent with a sterically restricted binding site, and a positive electronic effect of $\rho = 1.4$ on product dissociation, ascribed to a loss of product from an enzyme-bound Cu(II)–alkoxide complex. These results lead us to propose a mechanism in which O–O homolysis [from a putative Cu(II)–OOH species] and C–H homolysis (from substrate) occur in a concerted fashion, circumventing the formation of a discrete, high energy oxygen species such as hydroxyl radical. The substrate and peroxide-derived radical intermediates thus formed undergo a recombination, kinetically limited by displacement of an intervening water molecule, to give the postulated Cu(II)–alkoxide product complex.

Dopamine β-monooxygenase, a copper-containing enzyme, catalyzes the hydroxylation of dopamine to norepinephrine concomitant with the reductive cleavage of dioxygen. Despite its key role in neurotransmitter biosynthesis, the enzyme displays an unexpected lack of substrate specificity, catalyzing the hydroxylation of a variety of both 2- and aryl-substituted phenylethylamines (Kaufman & Friedman, 1965; May et al., 1981; Klinman & Krueger, 1982) and the benzylic oxidation of such groups as sulfides (May et al., 1981), olefins (May et al., 1983; Colombo et al., 1984), and aldehydes (Bossard & Klinman, 1985). As summarized in eq 1, an additional two electrons and two protons are required to complete the reduction; ascorbic acid is believed to be the physiologic reductant (Terland & Flatmark, 1975), but other one- and

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