# Mechanism-Based Inhibitors of Dopamine β-Hydroxylase: Inhibition by 2-Bromo-3-(*p*-hydroxyphenyl)-1-propene†

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ABSTRACT: The compound 2-bromo-3-(*p*-hydroxyphenyl)-1-propene (I) is a substrate of dopamine β-hydroxylase from adrenal medulla, and the product has been identified by mass spectrometry as 2-bromo-3-hydroxy-3-(*p*-hydroxyphenyl)-1-propene (II). Compound I also inactivates dopamine β-hydroxylase in a mechanism-based fashion. Thus, in acetate buffer at pH 5.0, inactivation by I exhibits saturation kinetics with a $K_D$ = 4.5 μM and $k_{inact}$ = 0.09 $min^{-1}$. The inactivation is strictly dependent on $O_2$ and a reducing agent (ascorbate or ferrocyanide) and is irreversible with no reactivation occurring upon prolonged dialysis or passage through a gel filtration column. The observed rate of inactivation at [I] = 4.5 mM (pH 5.5) increases from 0.045 to 0.17 $min^{-1}$ when $[O_2]$ is increased from 0.25 to 1.2 mM. Norepinephrine affords competitive protection against inactivation of enzyme by I. In initial velocity experiments, I is a linear competitive inhibitor vs. tyramine. The log $K_{is}$ vs. pH profile is flat while the log $k_{inact}$ vs. pH profile has an inflection corresponding to a group with a p$K_a$ of 5.7 ± 0.1. These data demonstrate that an enzymic group in its protonated form is involved in the inactivation reaction and not in the binding of I to the enzyme. In addition, inactivation requires a catalytically competent enzyme, inasmuch as no inactivation occurs when $Cu^{2+}$-free enzyme is incubated with I in the presence of ascorbate and $O_2$. At pH 5.5, as $Cu^{2+}$ is added to apoenzyme or to enzyme of low copper content, the rate of inactivation at saturating I increases and reaches a maximal value at eight $Cu^{2+}$ per tetramer. Because inactivation is strictly dependent on catalysis and follows saturation kinetics, i.e., a Michaelis complex is formed, we infer that eight Cu per tetramer are required for fully active enzyme and that four active sites each containing two $Cu^{2+}$ ions exist per tetramer. On the basis of these data, a mechanism of inactivation is suggested that involves enzyme-catalyzed activation of I to a reactive intermediate or to II followed by formation of a covalent adduct with the enzyme.

Dopamine β-hydroxylase (EC 1.14.17.1) is a copper-containing monooxygenase that requires an exogenous electron donor to catalyze the stereospecific hydroxylation at the benzylic carbon of many substituted phenylalkylamines (Kaufman & Friedman, 1965; Skotland & Ljones, 1979a; Rosenberg & Lovenberg, 1980). In catecholamine biosynthesis in vivo (Molinoff & Axelrod, 1971), dopamine β-hydroxylase catalyzes the conversion of dopamine to norepinephrine. From a pharmacological point of view, specific inhibitors of dopamine β-hydroxylase are potentially attractive as drugs to alter the production of catecholamines in vivo. Our interest in developing new substrates and inhibitors of this enzyme is not only because of their biological potential but also for their utility as new probes to study the mechanism of the hydroxylation reaction.

Previous work from our laboratory (Baldoni & Villafranca, 1980; Colombo et al., 1983a) has shown that a series of benzyl cyanide analogues of tyramine appeared to act as mechanism-based inhibitors of dopamine β-hydroxylase. The inactivation mechanism was complex (Colombo et al., 1983b) and involved several modes of inhibition depending upon the incubation conditions. Ideally, mechanism-based inactivators (suicide substrates or $k_{cat}$ reagents) are essentially inert as chemical reagents until a specific enzyme-catalyzed activation occurs (Rando, 1974, 1977; Abeles et al., 1976; Walsh, 1982). Thus, a chemically reactive form of the substrate analogue is produced in a specific environment, the active site of the target enzyme. Covalent bond formation can occur by subsequent reaction of this activated or chemically rearranged compound with a properly interposed residue at the active site. The specificity of the inactivation arises from both binding of the molecule and catalytic conversion to a reactive species prior to formation of a covalent adduct.

In a preliminary report, we demonstrated that 2-bromo-3-(*p*-hydroxyphenyl)-1-propene (I)[1] is a substrate for dopamine β-hydroxylase (Colombo et al., 1983c). This compound is hydroxylated by the enzyme to 2-bromo-3-hydroxy-3-(*p*-hydroxyphenyl)-1-propene (II) as shown in Scheme I. This paper presents a more complete description of I as a mechanism-based inhibitor of dopamine β-hydroxylase. Various kinetic criteria for inhibition by mechanism-based inhibitors have been applied in this study, and we conclude that I meets all these criteria. The mechanism of inhibition likely involves nucleophilic attack by a base at the active site of dopamine β-hydroxylase on an intermediate generated at the active site. Furthermore, we have taken advantage of the properties of the inactivation reaction to study the stoichiometry of the copper involved in the hydroxylation reaction. Our results suggest that eight Cu per tetramer are involved in catalysis and that there are four active sites per enzyme tetramer.

## Materials and Methods

*Materials.* Tyramine hydrochloride, disodium fumarate, ascorbic acid, MES, PIPES, ferrocyanide, (*R*)-norepinephrine, methyl α-mannoside, and potassium phosphate were from Sigma Chemical Co. Catalase (crystalline suspension in water, 65 000 units/mg) was from Boehringer, Bio-Gel P-6DG from Bio-Rad, DE-53 from Whatman, and concanavalin A–Se-

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[1] Abbreviations: I, 2-bromo-3-(*p*-hydroxyphenyl)-1-propene; II, 2-bromo-3-hydroxy-3-(*p*-hydroxyphenyl)-1-propene; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

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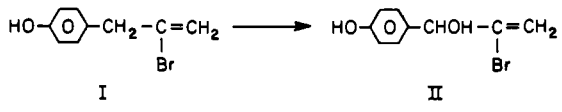
Scheme I



I II

pharose from Pharmacia. Gold-label DMF, acetate, and norpseudoephedrine were from Aldrich Chemical Co. EDTA was from Fisher. $CuCl_2 \cdot 2H_2O$ (puratronic grade) was from Alfa, and $CuSO_4$ (1000 ppm Cu) was from Scientific Products. Guanidine hydrochloride was from Schwarz/Mann, and $NaCNB^3H_3$ and $NaB^3H_4$ were from Amersham. All solutions were prepared with doubly distilled water run through a Millipore Super-Q ultrapure water system. 2-Bromo-3-(*p*-hydroxyphenyl)-1-propene was synthesized by coupling the organocuprate reagent prepared from *p*-bromoanisole with 2,3-dibromopropene. Thus, 22 mmol of $CuI \cdot (CH_3)_2S$ (House et al., 1975) was dissolved in 30 mL of ether and cooled to −10 °C under $N_2$, and 45 mmol of the Mg Grignard of *p*-bromoanisole was added with stirring. To this solution was added 22 mmol of 2,3-dibromopropene, and the reaction solution was allowed to warm to room temperature for about 4 h. Crude 2-bromo-3-(*p*-methoxyphenyl)-1-propene was extracted into ether and the solvent removed by rotoevaporation. Demethylation with $BBr_3$ was conducted in dry methylene chloride for 8 h at room temperature, and subsequent workup yielded pure I as established by NMR, mass spectrometry, and chromatography (Rajashekhar et al., 1984).

*Enzyme Preparation.* Dopamine β-hydroxylase was isolated from chromaffin granules of bovine adrenal medulla by chromatography on concanavalin A–Sepharose and DE-53 cellulose. The details of the modified purification procedure will be published elsewhere. The stock enzyme was stored at 4 °C as a suspension in ammonium sulfate. Prior to use, it was freed of ammonium sulfate by dialysis against 50 mM PIPES, pH 6.5. Four changes of buffer were made over a 36-h period. The first and second changes contained 10 and 1 μM $CuCl_2$, respectively. The last two changes employed buffer alone. Each dialysis change was against a 250-fold volume excess of buffer. This dialysis protocol resulted consistently in enzyme containing ca. eight Cu per tetramer. The dialyzed enzyme was stored at 4 °C and at a protein concentration greater than 1.5 mg/ml. The protein concentration was determined spectrophotometrically by using $\epsilon_{280}^{1\%} = 12.4$ (Skotland & Ljones, 1977). The protein concentration was calculated by using a tetramer $M_r$ of 290000 (Wallace et al., 1973).

*Enzyme Activity.* Dopamine β-hydroxylase was assayed polarographically with a Clark oxygen electrode and a YSI Model 53 biological oxygen monitor. The standard assay mixture in a final volume of 3 mL contained 200 mM sodium acetate, pH 5.0, 10 mM fumarate, 60 μg/mL catalase, 15 mM tyramine, and 20 mM ascorbate. Ascorbate (100 μL, freshly prepared solution) was added to each oxygraph cell just prior to the insertion of the oxygen electrode into the remainder of the assay mixture that had been equilibrated with air (0.22 mM $O_2$) by stirring at 37 °C. The assay was started by the addition of 3–10 μL of enzyme solution after the nonenzymatic endogenous rate for ascorbate oxidation had been established. All enzymatic rates were corrected for the nonenzymatic rate of ascorbate oxidation. Specific activity is expressed as micromoles of $O_2$ per minute per milligram of enzyme and our modified purification procedure yielded enzyme of specific activity 60–70.

*Enzyme Inactivation.* Dopamine β-hydroxylase was incubated at room temperature with I, $O_2$, and ascorbate as indicated in each legend. At various time intervals, aliquots were removed and assayed for enzyme activity in the standard assay at pH 5.0 and 37 °C. The dilution into the assay was 300–1000-fold. Controls in which the enzyme was incubated with ascorbate and no analogue or with analogue and no ascorbate were always run. All incubation mixtures contained a final concentration of 13.8% (v/v) DMF in which the analogue was dissolved. This concentration of DMF causes only a slight loss of enzyme activity when the time of incubations was shorter than 60 min if the temperature was 23–25 °C. Loss of enzyme activity does occur with 13.8% DMF when the incubation temperature is 37 °C.

*Copper Analysis.* The copper content of protein samples, assay mixtures, and incubation mixtures was determined by atomic absorption analysis with a Varian Techtron AA5 spectrometer equipped with a Model 63 carbon-rod atomizer and recorder. $CuSO_4$ was used to standardize the instrument.

*Apoenzyme Preparation.* Dopamine β-hydroxylase was dialyzed against 50 mM MES, pH 5.6, or 50 mM PIPES, pH 6.5, containing 10 mM EDTA followed by four changes of buffer in the absence of EDTA. Each change was against a 500-fold excess volume of buffer.

*Data Analysis.* The data for the pH–rate profile for inactivation of the enzyme by I were fitted by the least-squares analysis of Cleland (1979).

*Reduction of Control and Inactivated Dopamine β-Hydroxylase.* Dopamine β-hydroxylase (1 mg, two Cu per tetramer) was incubated in 100 mM MES, pH 5.5, containing 0.36 mg/mL catalase, 14% DMF, 5.1 mM I, and 32 mM ascorbate until ~10% of the enzyme activity remained. A control incubation solution (minus I) was treated similarly. These enzyme samples were then dialyzed against 250-fold excess 50 mM MES, pH 6.5 (four changes over 36 h), followed by chromatography on concanavalin A–Sepharose columns (0.4 $cm^3$) equilibrated with 50 mM potassium phosphate, pH 6.5, containing 200 mM NaCl. Catalase elutes with the equilibration buffer while dopamine β-hydroxylase remains bound. The latter was eluted with 10% methyl α-mannoside. Fractions containing dopamine β-hydroxylase were concentrated to 1 mg/mL and dialyzed against 100 mM potassium phosphate, pH 7.4. The activity of control and inactivated enzyme did not change during dialysis or chromatography. The $NaB^3H_4$ reduction of control and inactivated enzyme samples (2.4 μM tetramer) was carried out at room temperature for 30 min in the presence or absence of 2.6 M guanidine hydrochloride with 2.3 mM $NaB^3H_4$ (9.7 Ci/mmol) in 100 mM potassium phosphate, pH 7.0. Excess $NaB^3H_4$ was removed by chromatography on 3-$cm^3$ Bio-Gel P-6DG columns by employing the centrifugation method of Penefsky (1979). Analogous experiments were conducted with $NaCNB^3H_3$ (10 Ci/mmol) at pH 3.8 in acetate buffer. Incorporation of tritium into enzyme was determined by liquid scintillation counting in 10 mL of Optisol (Isolab) on a Beckman LS-7000 counter.

## Results

*Kinetics of Inactivation of Dopamine β-Hydroxylase by 2-Bromo-3-(p-hydroxyphenyl)-1-propene (I).* Dopamine β-hydroxylase from adrenal medulla catalyzes the conversion of I to II (Scheme I). The $k_{cat}$ value is 13.4 $min^{-1}$ at pH 5.5 in 100 mM acetate–MES buffer with 6.1 mM I, 1.2 mM $O_2$, and saturating ascorbate.[2] I also inactivates dopamine β-

[2] The kinetic parameters at saturating ascorbate, acetate–MES buffer, pH 5.5, are $k_{cat} = 0.19$ μmol $min^{-1}$ $mg^{-1}$, $K_m$ for I = 5.9 mM, and $K_m$ for $O_2$ = ~2.6 mM.

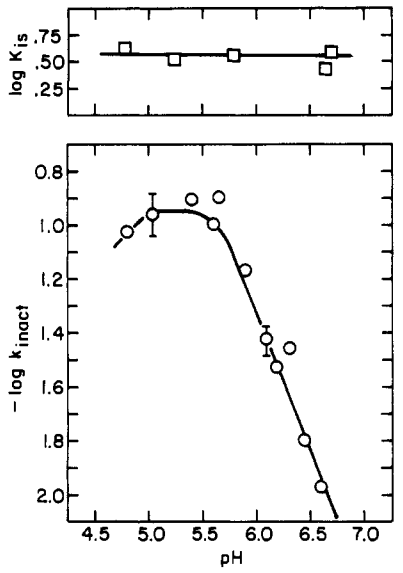



FIGURE 1: pH–rate profiles for $K_{is}$ and $k_{inact}$. The pH dependence of $K_{is}$ (upper panel) was determined at 26 °C by monitoring the rate of $O_2$ uptake as indicated under Materials and Methods for the standard assay. The 3-mL assay mixtures buffered with acetate–MES (0.1 M each, pH 4.8–6.7) also contained 14.3% DMF, 170 μg/mL catalase, 20 mM fumarate, 20 mM ascorbate, and 0.25 mM $O_2$. Tyramine was varied from 0.7 to 15 mM and I from 2 to 5.5 mM. The value for $K_{is}$ at each pH was determined from secondary plots of slope vs. inhibitor concentration. For the pH dependence of $k_{inact}$ (lower panel), dopamine β-hydroxylase (0.2 mg/mL) was incubated at room temperature in acetate–MES buffer (0.1 M each, pH 4.7–6.8) containing 13.8% DMF, 156 μg/mL catalase, 20 mM fumarate, 40 mM ascorbate, and 0.25 mM $O_2$. The concentration of I was varied from 0.9 to 4.5 mM. The value for $k_{inact}$ ($min^{-1}$) at each pH was determined from double-reciprocal plots of $k_{obsd}$ as a function of inhibitor concentration. The actual pH of the assay or incubation mixtures was determined with an Orion Research Model 601 A digital ion analyzer.

hydroxylase in a time-dependent manner only in the presence of both $O_2$ and ascorbate. A semilog plot of activity vs. time of incubation at various concentrations of I was linear for at least five half-lives (data not shown). From the slope of each line, the pseudo-first-order rate constant, $k_{obsd}$ was calculated. Consistent with the formation of a reversible complex prior to inactivation (eq 1), a plot of $1/k_{obsd}$ vs. $1/[I]$ (eq 2) was

$$E + I \rightleftharpoons E{\cdot}I \rightarrow E\text{–}I' \quad (1)$$

$$1/k_{obsd} = (K_D/k_{inact})(1/[I]) + 1/k_{inact} \quad (2)$$

linear with a finite intercept ($1/k_{inact}$) on the ordinate. $K_D$, the dissociation constant for the E·I complex, is also obtained from this plot (eq 2). At room temperature in acetate buffer at pH 5.0 and 0.25 mM $O_2$, the values for $k_{inact}$ and $K_D$ were respectively 0.09 $min^{-1}$ and 4.5 mM.

*pH Dependence of the Interaction of I with Dopamine β-Hydroxylase.* Under steady-state conditions, I is a linear competitive inhibitor vs. tyramine. Figure 1 (top) shows that the $K_{is}$ value does not change within experimental error from pH 4.8 to 6.7. The pH dependence of the inactivation of dopamine β-hydroxylase at infinite inhibitor concentration ($k_{inact}$) shows a linear portion with a slope of 1.1 (Figure 1, bottom). An enzymic group with a $pK_a$ of 5.7 ± 0.1 is suggested from the inflection in this plot. Inspection of the data in Figure 1 (bottom) appears to indicate that an additional group with a $pK_a < 5$ might also be required for inactivation. However, the enzyme was too unstable under the conditions of this experiment at pH values lower than 4.5 to obtain reliable kinetic data.

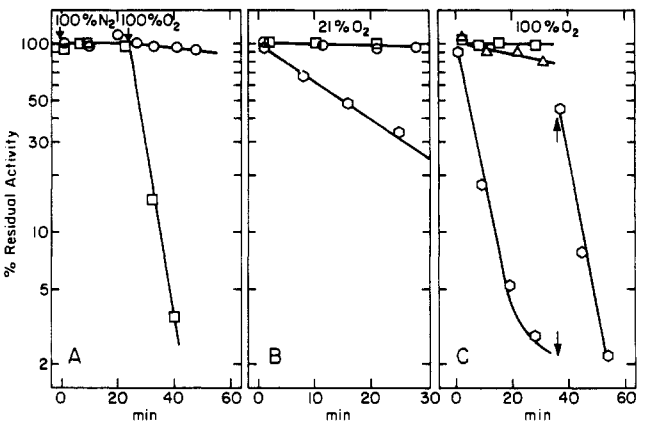



FIGURE 2: Effect of substrates on the inactivation of dopamine β-hydroxylase with 2-bromo-3-(*p*-hydroxyphenyl)-1-propene. Dopamine β-hydroxylase (0.25 mg/mL) was incubated at room temperature in 100 mM each acetate–MES, pH 5.5, containing 13.8% DMF, 20 mM fumarate, 38 mM ascorbate, and 4.5 mM compound I [(□) panel A; (○) panels B and C] and also 30 mM tyramine [(△) panel C]. Control incubations were run in the absence of ascorbate [(○) panels A and B] or in the absence of compound I [(□) panels B and C]. In panel A, the incubations started under 100% $N_2$ and were switched to 100% $O_2$ at the time indicated by the arrow. The incubations in panel B were with 21% $O_2$ (0.25 mM) and in panel C were with 100% $O_2$ (1.2 mM). At the time indicated by the arrow in panel C, a second aliquot of enzyme was added, and the incubation was continued.

*Effect of Substrates of Dopamine β-Hydroxylase on Inactivation by I.* The data in Figure 2 show the effect of varying the concentration of $O_2$ upon inactivation with I. In the absence of oxygen ($N_2$ atmosphere), no inactivation of dopamine β-hydroxylase occurred even though a reducing agent and I were present. But when this incubation mixture becomes saturated with 100% oxygen, rapid inactivation occurs (Figure 2A, squares). In a control incubation solution in the absence of ascorbate under either 100% nitrogen or 100% oxygen (Figure 2A, circles), dopamine β-hydroxylase is not inactivated by I. Increasing the concentration of oxygen from 21 to 100% $O_2$ results in a 4-fold increase in the inactivation rate (Figure 2B,C). No inactivation results if dopamine β-hydroxylase is incubated with oxygen and I or with oxygen and ascorbate (Figure 2B, controls).

If I is inhibiting the enzyme at or near the active site, substrates and inhibitors that bind at the active site should slow the rate of inactivation. Indeed, the presence of 20 mM tyramine results in a 25-fold increase of the $t_{1/2}$ for inactivation (Figure 2C, hexagons vs. triangles). (*R*)-Norepinephrine, a competitive inhibitor of the enzymatic reaction (see below), also protects the enzyme against inactivation by I in a concentration-dependent manner (Figure 3). The inset in Figure 3 is a plot of $1/k_{obsd}$ vs. [inhibitor], which is analogous to a Dixon plot for competitive inhibition. The intercept of this plot gives a $K_i$ value of 48 mM, in good agreement with a $K_{is}$ value of 40 mM obtained in an independent steady-state kinetic experiment using tyramine as substrate. Other replots[3] confirm the competitive nature of the inhibition.

Because the data in Figure 2C do not show a lag in the onset of inhibition, the active inhibitor species is not released from

[3] The competitive kinetic relationship between I and (*R*)-norepinephrine was confirmed upon plotting the data in Figure 3 as $1/k_{obsd}$ vs. $1/[I]$ in the absence and presence of (*R*)-norepinephrine. This plot gives a pattern intersecting on the vertical axis at the $1/k_{inact}$ value. Furthermore, a plot of $[I]/k_{obsd}$ vs. [(*R*)-norepinephrine] results in a set of parallel lines characteristic of a competitive relationship. Noncompetitive, uncompetitive, or mixed type of kinetic relationships result in intersecting sets of lines (Segel, 1975).

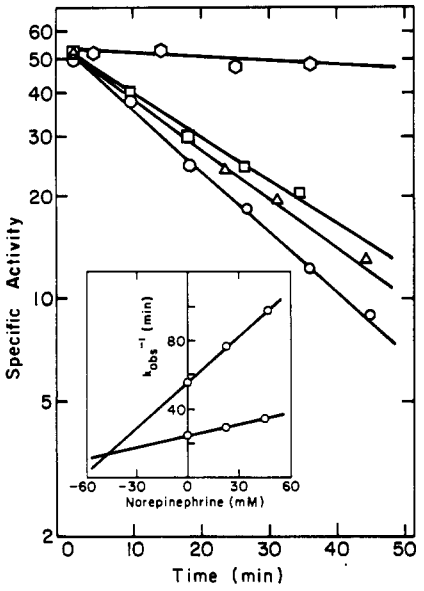



FIGURE 3: Protection by norepinephrine of inactivation of dopamine β-hydroxylase by 2-bromo-3-(*p*-hydroxyphenyl)-1-propene. Dopamine β-hydroxylase (244 μg/mL) was incubated at room temperature in 200 mM acetate, pH 5.0, containing 13.8% DMF, 156 μg/mL catalase, 10 mM fumarate, and 38 mM ascorbate in the presence of 3.5 mM I alone (○) and with 22.5 (△) or 45 mM (□) (*R*)-norepinephrine. The control incubation (○) contained ascorbate and 45 mM norepinephrine but no I. An identical set of incubations with 1 mM I was also run (not shown). (Inset) Reciprocal of the pseudo-first-order rate constants for inactivation ($k_{obsd}$) vs. the concentration of norepinephrine at each concentration of I. The intercept to the left of the ordinate gives the $K_i$ for (*R*)-norepinephrine.

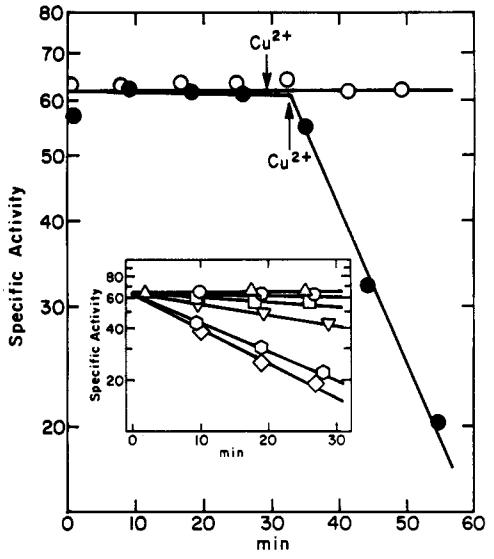



FIGURE 4: Effect of copper on the inactivation of dopamine β-hydroxylase by 2-bromo-3-(*p*-hydroxyphenyl)-1-propene. Apo dopamine β-hydroxylase (1 μM tetramer, 0.3 Cu per tetramer) was incubated at room temperature in 100 mM MES, pH 5.5, containing 13.8% DMF, 156 μg/ml catalase, 0.25 mM $O_2$, 4.5 mM compound I (○), and 38 mM ascorbate (●). At the time indicated by the arrows, $CuCl_2$ at a final concentration of 8 μM was added to each incubation. The inset shows the effect of increasing amounts of added $CuCl_2$ [(○) 0.3 μM; (□) 2.2 μM; (▽) 4.2 μM; (○) 8.1 μM; (◇) 11.9 μM) on the $k_{obsd}$ for the turnover-dependent inactivation of the enzyme by I under identical conditions as described above. The control (△) contained no ascorbate and was run at the highest copper concentration. In the absence of dopamine β-hydroxylase, the copper control of the incubation solution (minus ascorbate) was determined by atomic absorption to be 0.2 μM.

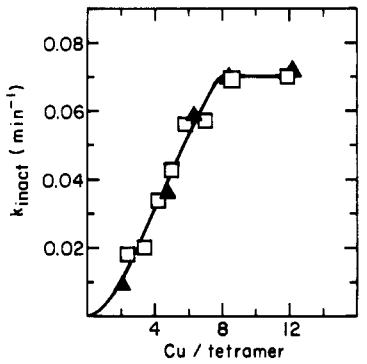



FIGURE 5: Copper dependence of the rate constant for inactivation at infinite inhibitor concentration ($k_{inact}$). Dopamine β-hydroxylase (1.1 μM tetramer) containing 1.9 (▲) or 2.2 (□) Cu per tetramer was incubated at room temperature in 100 mM MES, pH 5.6, containing 13.8% DMF, 156 μg/ml catalase, 0.25 mM $O_2$, 38 mM ascorbate, and I. The concentration of I was varied from 0.9 to 4.5 mM at each copper level. The ratio of Cu to dopamine β-hydroxylase tetramer was adjusted by adding $CuCl_2$ to the incubation mixtures containing 1.1 μM enzyme. The $k_{inact}$ value was calculated from plots of $1/k_{obsd}$ vs. $1/[I]$ at each Cu to tetramer ratio.

the enzyme prior to inhibition. This is further proven by the observation that an aliquot of fresh enzyme can be added to an incubation solution in which >98% of the dopamine β-hydroxylase activity had been lost and the $t_{1/2}$ for inactivation is identical in the first and second inactivation cycles (Figure 2C, hexagons). Thus, direct inactivation of the enzyme is occurring as expected for a mechanism as in eq 1.

*Copper Dependence of the Inactivation Reaction.* The experiments described above show that $O_2$ and ascorbate are required for inactivation of dopamine β-hydroxylase by I and imply that enzymatic conversion of I to an intermediate or to product precedes the inactivation step. If this is the case, anything that blocks catalysis should also prevent inactivation from occurring. We decided to test if inactivation occurs with $Cu^{2+}$-free enzyme because the apoenzyme is catalytically inactive (Skotland & Ljones, 1979b; Blackburn et al., 1980; Lovenberg et al., 1975). Figure 4 (filled circles) shows that apoenzyme incubated with oxygen, ascorbate, and I for 30 min does not lose appreciable activity ($t_{1/2}$ for inactivation > 1300 min). However, when copper is added in a ratio of eight per tetramer to this incubation solution, the $t_{1/2}$ for inactivation is 13 min. A control enzyme was stable during this same incubation time (Figure 4, open circles). Under these conditions, the rate of inactivation was dependent on the copper to enzyme ratio and appeared to level off at about 8–12 Cu per tetramer (Figure 4, inset). At this point, it is important to consider the experimental protocols. The copper content of the incubation mixtures (without enzyme and ascorbate) was determined to be 0.2–0.3 μM by atomic absorption analysis, and with 1 μM enzyme, the enzyme exceeds the amount of copper in the buffer. The amount of copper in the buffer was included in the calculations of total Cu in each experiment.[4]

We studied the copper dependence of the inactivation reaction in detail. Inasmuch as inactivation by I occurs during catalysis and follows saturation kinetics, the rate of inactivation

[4] In the standard assay at pH 5.0 in a 3-mL volume (see Materials and Methods), the specific activity is the same for apoenzyme and holoenzyme. This assay mixture (minus ascorbate and enzyme) contains ~0.5 μM Cu as determined by atomic absorption analysis. Thus, if a 3-μL aliquot of the incubation solution containing 1 μM enzyme tetramer is transfered into the 3-mL assay solution, the final copper to enzyme ratio is 500:1. Therefore, enough copper is present in the assay solution to saturate the enzyme when it is transfered from the incubation mixtures no matter what the initial enzyme to copper ratio is in the various incubation solutions.

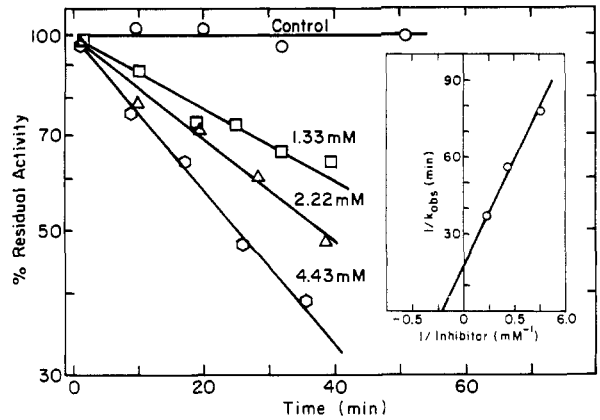



FIGURE 6: Time course of the inactivation of dopamine β-hydroxylase with 2-bromo-3-(*p*-hydroxyphenyl)-1-propene and dependence of the pseudo-first-order rate constant of inactivation ($k_{obsd}$) on analogue concentration. Dopamine β-hydroxylase (0.18 mg/mL) was incubated at room temperature and under air saturation in 110 mM MES buffer, pH 5.5, containing 13.8% DMF, 25 μM $CuCl_2$, 164 μg/mL catalase, and 4.5 mM I in the absence of ferrocyanide [(○) control] or with 0.125 mM ferrocyanide and I at the indicated concentration (○, △, and □). (Inset) Double-reciprocal plot of $k_{obsd}$ vs. concentration of I.

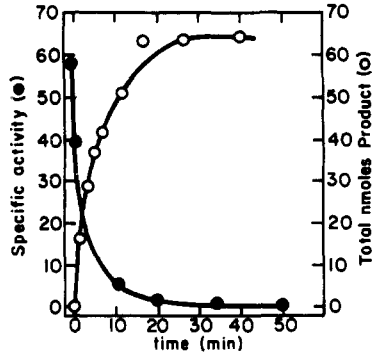



FIGURE 7: Partition between enzyme inactivation and turnover. Dopamine β-hydroxylase (1.76 nmol of monomer) was incubated at room temperature and at pH 5.5 in acetate–MES (0.1 M each) containing 13.8% DMF, 156 μg/mL catalase, 20 mM fumarate, 100% $O_2$, 38 mM ascorbate, and 6.1 mM I. Residual enzyme activity (●) was determined in the standard assay at pH 5.0. Product formed (○) was determined by HPLC on a Waters μBondapak $C_{18}$ column. Reverse-phase chromatography was carried out with 60% 50 mM acetic acid and 40% $CH_3CN$ as the mobile phase (v/v). The product II eluted with a retention time of 2.6 min.

at saturating I was measured as a function of the Cu to tetramer ratio. This is analogous to a study of $V_{max}$ vs. [Cu]. Figure 5 shows that $k_{inact}$ increases with increasing Cu and then reaches a maximum at a ratio of eight Cu per tetramer. Half-maximal inactivation is obtained at a Cu to enzyme ratio of ~4. These data were obtained with two different enzyme preparations containing 1.9 and 2.2 Cu per tetramer (Figure 5, triangles and squares, respectively). Apoenzyme prepared as described under Materials and Methods was not used in these experiments because even after exhaustive dialysis, the enzyme still retained some EDTA (unpublished results).

The data in Figures 2, 4, and 5 taken together strongly suggest that catalytic conversion of I to an enzyme-bound intermediate or product precedes the inactivation step. Thus, I is not a simple affinity label of dopamine β-hydroxylase.

*Inactivation of Dopamine β-Hydroxylase in the Presence of Ferrocyanide.* Ferrocyanide can replace ascorbate in the normal assay with tyramine as substrate (Rosenberg & Lovenberg, 1980). Dopamine β-hydroxylase was incubated with I and ferrocyanide, and the data are presented in Figure 6. Inactivation is also observed with ferrocyanide in place of ascorbate, and the inactivation follows saturation kinetics with a $K_D$ of 4.6 mM for I and $k_{inact}$ = 0.06 $min^{-1}$. By comparison, $K_D$ = 3.6 mM and $k_{inact}$ = 0.071 $min^{-1}$ with ascorbate under nearly identical conditions (Figure 5). No inactivation was observed with I or with ferrocyanide alone.

*Partition of Turnover vs. Inactivation.* The data in Figure 7 show that when 1.76 nmol of dopamine β-hydroxylase (monomer) is incubated with $O_2$, ascorbate, and I, a >98% loss of enzyme activity is achieved when 65 nmols of product is present in solution. Hence, the ratio of turnover vs. inactivation is 36. This partition ratio is independent of the concentration of I (not shown). This ratio was also calculated from the turnover and inactivation parameters. In a typical inactivation experiment at pH 5.5, 25 °C, and 1.2 mM $O_2$, the $k_{inact}$ value is 0.38 $min^{-1}$. Under identical experimental conditions, the $k_{cat}$ value obtained from steady-state kinetics is 13.4 $min^{-1}$. The partition ratio ($k_{cat}/k_{inact}$) is then equal to 35. This indicates that the minimal inactivation mechanism in eq 1 should be expanded to eq 3 for a more complete description of the inactivation events.

$$E + S \rightleftharpoons E \cdot S \longrightarrow E \cdot P \begin{matrix} \nearrow E + P \\ \searrow E_{inact} \end{matrix} \quad (3)$$

*Incubation of Dopamine β-Hydroxylase with II.* Because inactivation of dopamine β-hydroxylase by I occurs only during catalysis, one should consider the possibility that the reactive species responsible for inactivation is the product, II. Thus, if one were to incubate the enzyme with II in the absence of ascorbate, inactivation may occur. Inasmuch as the product of the hydroxylation reaction with the normal substrate is the *R* isomer (Taylor, 1974; Bacham et al., 1974; Battersby et al., 1974, 1976; May et al., 1981), we enzymatically synthesized II by incubating I with dopamine β-hydroxylase, ascorbate, and 100% oxygen. The hydroxylation product was extracted with ether, concentrated, and characterized by HPLC and mass spectrometry. Upon incubation of dopamine β-hydroxylase at pH 5.5 with 2.2 mM enzymatically synthesized II, significant enzyme inactivation occurred. The observed rate of inactivation was 0.002 $min^{-1}$. The limited quantities of II that were available precluded the determination of a value for $k_{inact}$ and of a dissociation constant for the enzyme–product complex. Nonetheless, this experiment clearly showed that II (presumably the *R* isomer) is capable of interacting at the active site of the enzyme to inactivate dopamine β-hydroxylase. No inactivation was detectable when the enzyme was incubated with II in the presence of ascorbate, indicating that the oxidized form of the enzyme reacts with II.

We estimated the affinity of II for dopamine β-hydroxylase by comparison with the relative affinity of the substrate–product pair dopamine–norepinephrine for the enzyme. We undertook steady-state kinetic studies in order to determine the relative affinity of dopamine and norepinephrine by comparing the values of the $^{app}K_m$ (apparent $K_m$) and $K_{is}$ for substrate and product, respectively. The $^{app}K_m$ for dopamine is 5.8 mM. Norepinephrine (up to 40 mM) was a linear competitive inhibitor vs. tyramine with a $K_{is}$ value of 40 mM. These data indicate that II probably binds very poorly to dopamine β-hydroxylase and, hence, may be one reason for the low inactivation rate observed at 2.2 mM II (see above).

We also investigated norpseudoephedrine as an inhibitor vs. tyramine. This compound is an α-methyl-β-hydroxy analogue of the normal product and of II and is an inhibitor at pH 5.0 (see Materials and Methods) with a $K_{0.5}$ = 170 mM at 1.5 mM tyramine.

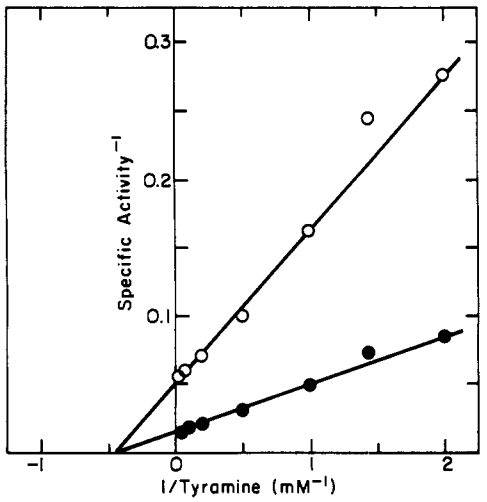



FIGURE 8: Double-reciprocal plots for control and partially inactivated dopamine β-hydroxylase. The steady-state assays were at pH 5.0 and 37 °C as indicated under Materials and Methods for the standard assay. Tyramine was the variable substrate. The enzymes are (●) control and (○) partially inhibited after dialysis against four changes of 50 mM PIPES, pH 6.5.

*Irreversibility of the Inactivation Reaction and Properties of Partially Inactivated Enzyme.* Dopamine β-hydroxylase was incubated for 33 min with I, oxygen, and ascorbate. At this time, 37% of the activity remained, and the inactivation reaction was halted by addition of a large excess of tyramine. A control enzyme solution (no I) was treated similarly. These enzymes were then dialyzed against 50 mM PIPES, pH 6.5, for 48 h (four changes of a 500-fold volume excess of buffer). The partially inactivated enzyme failed to regain catalytic activity. The control enzyme retained all of its original activity upon dialysis. When the activity of these two enzymes (control and partially inactivated) was determined in steady-state kinetic assays at pH 5.0 with tyramine as substrate, no change in the $^{app}K_m$ values for tyramine was observed (Figure 8). However, the $^{app}V_{max}$ values of the partially inactivated enzyme and control enzyme were respectively 20 and 63 μmol $min^{-1}$ $mg^{-1}$. These results indicate that partially inactivated enzyme is actually a mixed population of totally active and totally inactive dopamine β-hydroxylase.

No reactivation was found when aliquots of an enzyme solution undergoing inactivation were passed through a Bio-Gel column to remove small molecules by the method of Penefsky (1979). In Figure 9, a plot is presented of percent enzyme activity before and after gel filtration at various concentrations of I.

From other work in our laboratory, we showed that dopamine β-hydroxylase inactivated with benzyl cyanide substrate analogues could be reactivated by decreasing the pH of the incubation solution in the presence of a variety of anions (Colombo et al., 1983d). Because of these results, reactivation experiments of dopamine β-hydroxylase undergoing inactivation with I were attempted. For this purpose, dopamine β-hydroxylase was incubated at 25 °C at pH 5.5 with 0.25 mM $O_2$, 38 mM ascorbate, and 4.6 mM I. After a 28-min incubation, the enzyme had lost ~40% of its activity. At this time, the pH of the incubation solution was decreased to 4.7 by addition of acetate to a final concentration of 200 mM, and the incubation was continued at the lower pH. No reactivation of the enzyme occurred, but the observed rate of inactivation changed from 0.019 $min^{-1}$ at pH 5.5 to 0.033 $min^{-1}$ at pH 4.7. This general trend is consistent with the pH dependence of the inactivation reaction (Figure 1).

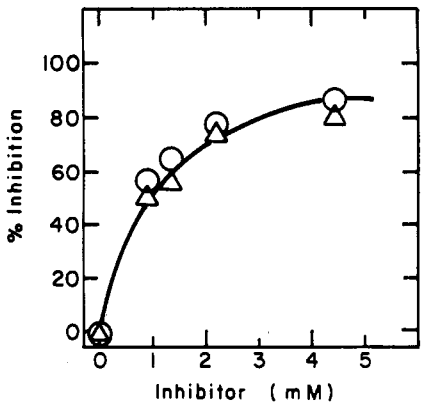



FIGURE 9: Irreversibility of the inactivation of dopamine β-hydroxylase by I. The incubation mixtures (250 μL) contained 0.29 mg/mL enzyme, 100 mM MES, pH 5.5, 13.8% DMF, 0.156 mg/mL catalase, 0.25 mM $O_2$, 38 mM ascorbate (control), and I at the indicated concentrations. After 50 min of incubation at room temperature, 4-μL aliquots were removed and assayed for enzymatic activity, and 230-μL aliquots were desalted by the method of Penefsky (1979) with a 3-$cm^3$ syringe filled with Bio-Gel P-6DG. After the gel filtration enzyme activity was redetermined, percent inhibition [before (○) and after (△) filtration] was calculated as the difference between 100 and the ratio of the activity of the modified enzyme to that of the unmodified control enzyme multiplied by 100. The concentrations of I were 0 (control) and 0.9–4.5 mM (modified).

*Reduction Experiments with Control and Inactivated Dopamine β-Hydroxylase.* Experiments were designed to test for the possible formation of a carbonyl moiety or Schiff base resulting from the mechanism-based inactivation of dopamine β-hydroxylase by I. Enzyme was incubated with I by the procedure presented under Materials and Methods and inactivated to 10% residual activity. A control enzyme sample was incubated in the absence of I. Separate samples were subjected to $NaCNB^3H_3$ reduction at pH 3.8, $NaB^3H_4$ reduction at pH 7.0 (nondenaturing conditions), or $NaB^3H_4$ reduction at pH 7.4 (denaturing conditions with guanidine hydrochloride). The inactivated enzyme contained only 2% of the expected tritium incorporation for a 90%-inhibited enzyme, assuming four active sites per tetramer.

## Discussion

The minimum kinetic criteria that a compound must meet to be considered a mechanism-based inhibitor are listed below. The results reported herein indicate that I meets all these criteria: (a) the inactivation is first order and follows saturation kinetics (Figures 3 and 6); (b) substrates or competitive inhibitors protect against inactivation (Figures 2 and 3); (c) the inactivation is complete, i.e., the $V_{max}$ alone changes for partially inactivated enzyme (Figures 2 and 8); (d) activity does not return upon exhaustive dialysis or passage through a gel filtration column (Figure 9); (e) a catalytically competent enzyme is required for inactivation (Figures 4 and 5); (f) partitioning between normal turnover and inactivation is observed (Figure 7). These data indicate a kinetic mechanism in which I irreversibly inactivates dopamine β-hydroxylase due to its enzymatic conversion to an enzyme-bound intermediate or product prior to inactivation.

In a preliminary paper we demonstrated that I is an alternate substrate for dopamine β-hydroxylase (Colombo et al., 1983c). The kinetic parameters obtained with I were $^{app}K_m$ = 5.9 mM and $k_{cat}$ = 13.4 $min^{-1}$ (pH 5.5, 100% $O_2$, 25 °C). Under identical conditions, preincubation of this substrate analogue with dopamine β-hydroxylase leads to a time-dependent loss of enzyme activity ($K_D$ = 3.9 mM and $k_{inact}$ = 0.38 $min^{-1}$). This inactivation is strictly dependent on catalysis

and is consistent with a mechanism-based inactivation of the enzyme (Rando, 1974; Abeles & Maycock, 1976; Walsh, 1982).

Other laboratories have also reported mechanism-based inhibitors of dopamine β-hydroxylase. Kinetic parameters for the hydroxylation of β-chlorophenethylamine (Klinman & Krueger, 1982) were $^{app}K_m$ = 5.1 mM and $k_{cat}$ = 66 min$^{-1}$ (pH 6, 21% $O_2$, 35 °C) and for the hydroxylation of 1-phenyl-1-(aminoethyl)ethene (May et al., 1983) were $^{app}K_m$ = 8.3 mM and $k_{cat}$ = 60 min$^{-1}$ (pH 5, 21% $O_2$, 37 °C). Incubation of dopamine β-hydroxylase with these analogues under catalytic conditions also leads to enzyme inactivation with $K_D$ = 4.4 mM and $k_{inact}$ = 56.4 × 10$^{-4}$ min$^{-1}$ for β-chlorophenethylamine and $K_D$ = 13 mM and $k_{inact}$ = 0.04 min$^{-1}$ for 1-phenyl-1-(aminoethyl)ethene. In terms of the relative specificity as suicide substrate for dopamine β-hydroxylase, I appears to be the most efficient so far. This efficiency is evaluated by the partition ratio, i.e., how often the normal hydroxylation cycle is completed ($k_{cat}$) compared to how often it is rerouted into irreversible inactivation of the enzyme ($k_{inact}$). The $k_{cat}/k_{inact}$ ratios are 12000 for β-chlorophenethylamine (Klinman & Krueger, 1982), 750 for 1-phenyl-1-(aminoethyl)ethene[5] (May et al., 1983), and 36 for I (this paper). I is also a more efficient suicide inhibitor of dopamine β-hydroxylase than the benzyl cyanide analogues previously reported by us. The $k_{cat}/k_{inact}$ ratio for *p*-hydroxybenzyl cyanide (pH 5, 21% $O_2$, 37 °C) was 8000 (Baldoni & Villafranca, 1980). For benzyl cyanide and *m*-hydroxybenzyl cyanide (pH 5.4, 21% $O_2$, 25 °C), the partition ratio was 219 and 347, respectively (Colombo et al., 1983a). The $k_{cat}/k_{inact}$ value for I compares also very favorably with the values reported with olefinic suicide substrates for other enzymes [Table 4 in Walsh (1982)].

The inactivation of dopamine β-hydroxylase by I is strictly dependent on enzymatic catalysis. No loss of enzyme activity occurs when dopamine β-hydroxylase is incubated with the analogues plus $O_2$, with ascorbate plus $O_2$, or with the analogue plus ascorbate plus $N_2$. Inactivation arises only when dopamine β-hydroxylase is incubated with the analogue, reducing agent (ascorbate or ferrocyanide), and oxygen. This fact and the similarity of $K_D$ values derived from enzyme inactivation ($K_D$ = 3.9 mM), substrate hydroxylation ($^{app}K_m$ = 5.9 mM), and steady-state competitive inhibition studies ($K_{is}$ = 4.9 mM) indicate that inactivation occurs in the course of or after the catalytic conversion of I to II but prior to product release. The notion that the enzyme could be producing its own affinity label is ruled out by the fact that the time course of the inactivation does not exhibit a lag (Figures 2–6) and by the observation that addition of a fresh aliquot of enzyme to an incubation in which inactivation had proceeded almost to completion results in a second inactivation cycle with a $t_{1/2}$ for inactivation identical with the one obtained in the first cycle (Figure 2C). Hence, the hydroxylation product does not diffuse into solution to later return and react with an active site residue.

Additional evidence that enzymatic conversion of I to a reactive compound or intermediate must precede inactivation arises from the fact that incubation of apoenzyme with the analogue, ascorbate, and oxygen does not result in enzyme inactivation (Figure 4). By analyzing the copper dependence of the inactivation reaction, we could obtain information about the Cu stoichiometry of the catalytic reaction. The rationale was as follows. (1) A fundamental aspect of the kinetics of mechanism-based inhibition (Abeles & Maycock, 1976; Walsh, 1982) is that a Michaelis complex is formed followed by a first-order inactivation reaction (E + S ⇌ E·S → E·P → $E_{inact}$). Thus the rate of inactivation when the enzyme is saturated is a measure of the catalytic activity. (2) Incubation experiments with I could be conducted with enzyme concentrations well above the level of contaminating copper in the incubation solution. We used enzyme with a Cu to tetramer ratio of ~2 to begin such studies and added additional $Cu^{2+}$ to the incubation solutions.

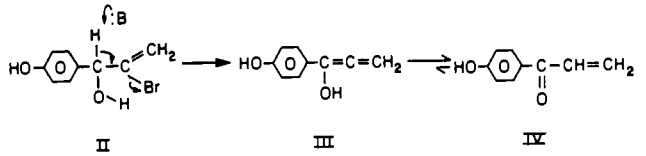
Our data show that the rate of inactivation at infinite inhibitor concentration ($k_{inact}$) is dependent on the Cu to tetramer ratio, reaches a half-maximal value at ca. four Cu per tetramer, and reaches a maximal value when 8 equiv of copper are present per dopamine β-hydroxylase tetramer (Figure 5). Even though isolated dopamine β-hydroxylase has been reported to contain 2–10 Cu per tetramer (Friedman & Kaufman, 1966; Goldstein et al., 1965; Ljones et al., 1976; Walker et al., 1977), it is not yet known how many active sites there are and how many coppers per active site are necessary for maximal activity. Because turnover precedes inactivation (see above) and two electrons per one $O_2$ per one substrate are consumed during the reaction (Friedman & Kaufman, 1965), presumably all eight Cu per tetramer are required for catalysis. The stoichiometry suggests that there are two Cu per active site if each subunit is active. Kaufman originaly suggested that there were two Cu per active site on the basis of a stoichiometry of two Cu per tetramer and proposed that the tetramer had one active site. Next, Skotland and Ljones proposed that each active site had one Cu on the basis of a stoichiometry of four Cu per tetramer. The copper dependence of the reaction was also studied by Blackburn et al. (1980), but their experiments were only extended to a Cu to tetramer ratio of ~2. However, their data suggested cooperativity in the binding of copper ions, which was interpreted as evidence for binuclear Cu centers. On the basis of our early NMR and EPR experiments, we proposed that each active site had two Cu per tetramer, but we also discussed several other alternative mechanisms (Villafranca, 1981). The data in this paper extended to a Cu to tetramer ratio of ~12 support the idea that the active tetramer has four active sites and two Cu per tetramer. Whether the curvature in the initial part of Figure 5 represents cooperativity in $Cu^{2+}$ binding or formation of binuclear centers remains to be determined.

The failure to recover enzymatic activity following gel filtration or prolonged dialysis of I-inhibited dopamine β-hydroxylase is consistent with inactivation arising from covalent enzyme modification. Several lines of evidence support the view that inactivation occurs at the active site. First, I is a substrate and a competitive inhibitor vs. tyramine with comparable $K_m$ and $K_{is}$ values. Second, in incubation experiments, tyramine or norepinephrine protect dopamine β-hydroxylase against turnover-dependent inactivation by I (Figures 2 and 3). The norepinephrine protection is concentration dependent, and the $K_D$ value of 48 mM (Figure 3) is in agreement with the $K_{is}$ value of 40 mM determined from the competitive inhibition studies. Third, the kinetic properties of partially inactivated dopamine β-hydroxylase, i.e., decreased $^{app}V_{max}$ value but identical $^{app}K_m$ value for tyramine (Figure 8), imply that the active site is probably modified by the inactivation process.

Even though we could not determine a $k_{inact}$ value for inactivation of dopamine β-hydroxylase by the product, II, we demonstrated that a significant loss of enzyme activity occurred upon incubation of the enzyme with 2.2 mM II, the highest concentration available for testing. This concentration of II

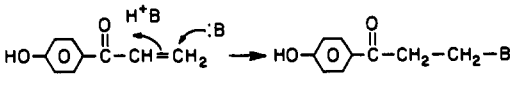
[5] Calculated from the rate constants given in May et al. (1983).

Scheme II



II III IV

Scheme III



IV V

is probably well below that required for saturation. We estimated the $K_D$ for II to be greater than 40 mM by comparison to the relative affinities of the substrate–product pair dopamine–norepinephrine.

The pH dependence of the inactivation reaction shows a group with a p$K_a$ of 5.7 in the basic side of the pH profile and perhaps a group with a p$K_a$ < 5 in the acid side of the profile (Figure 1, bottom). These p$K_a$ values correspond to enzymatic groups involved in the inactivation reaction and not residues involved in substrate binding because there are no p$K_a$ values expressed in the log $K_{is}$ vs. pH profile for I (Figure 1, top). Enzyme groups having p$K_a$ values between 5 and 6 have been found to be involved in dopamine β-hydroxylase catalyzed hydroxylation of dopamine (Ahn & Klinman, 1983) and of tyramine and benzyl cyanide substrate analogues (Colombo et al., 1983a).

Several mechanisms could account for the inactivation reaction. If the inactivation reaction with I occurs by a mechanism involving the enzyme-bound product II, one possibility involves loss of HBr resulting in formation of an allene (III), or its tautomer, an α,β-unsaturated ketone (IV) (Scheme II). Dopamine β-hydroxylase inactivation could then arise from nucleophilic attack on the central carbon of the allene or from a Michael-type addition on the terminal carbon of the α,β-unsaturated ketone. For this mechanism to occur, two groups at the active site of the enzyme are likely to be involved: a base to act as a nucleophile and an acid for protonation of the allene or vinyl ketone (Scheme III). This inactivation scheme predicts that an enzyme adduct (V) containing a carbonyl group would be formed. We tested for such an adduct by incubating 90%-inactivated enzyme with $NaCNB^3H_3$ at pH 3.8 or with $NaB^3H_4$ at pH 7.0 under nondenaturing conditions and at pH 7.4 with $NaB^3H_4$ under denaturing conditions with guanidine hydrochloride. In none of the experiments could we detect significant tritium incorporation into the enzyme. This experiment casts doubt on the inactivation mechanism presented above unless the putative carbonyl group reacts with other enzyme residues and survives the denaturation procedure. However, this seems unlikely.

Another reasonable mechanism would involve inactivation by rearrangement of an intermediate formed on the normal catalytic reaction pathway. From experiments measuring the deuterium isotope effects on $V_{max}$ and $V/K$ with a series of ring-substituted phenethylamines and benzyl cyanides, we suggest that a radical cation is formed during catalysis (B. Rajashekhar and J. J. Villafranca, unpublished results). Inactivation could occur by nucleophilic attack on an electrophilic species generated from the radical cation. It is too early to speculate on how inactivation of dopamine β-hydroxylase may proceed by such a mechanism. In sum, I has been demonstrated to be an irreversible inhibitor of dopamine β-hydroxylase that requires catalytic turnover by the enzyme. The enzyme-bound intermediate or product may rearrange to an electrophilic species prior to diffusion into solution and lead to formation of a stable adduct. Further evidence of the nature of the bound chemical species is being sought in our laboratory.

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References

Abeles, R. H., & Maycock, A. L. (1976) *Acc. Chem. Res. 9*, 313–319.

Ahn, N., & Klinman, J. B. (1983) *Biochemistry 22*, 3096–3106.

Bacham, L., Storm, C. B., Wheeler, J. W., & Kaufman, S. (1974) *J. Am. Chem. Soc. 96*, 6799–6800.

Baldoni, J., & Villafranca, J. J. (1980) *J. Biol. Chem. 255*, 8987–8990.

Battersby, A. R., Sheldrake, P. W., Staunton, J., & Williams, D. C. (1974) *J. Chem. Soc., Chem. Commun.*, 566–567.

Battersby, A. R., Sheldrake, P. W., Staunton, J., & Williams, D. C. (1976) *J. Chem. Soc., Perkin Trans. 1*, 1056–1062.

Blackburn, N. J., Mason, H. S., & Knowles, P. F. (1980) *Biochem. Biophys. Res. Commun. 95*, 1275–1281.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Colombo, G., Rajashekhar, B., Giedroc, D., & Villafranca, J. J. (1983a) *J. Biol. Chem. 259*, 1593–1600.

Colombo, G., Rajashekhar, B., Ash, D. E., & Villafranca, J. J. (1983b) *J. Biol. Chem. 259*, 1607–1615.

Colombo, G., Rajashekhar, B., & Villafranca, J. J. (1983c) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 407.

Colombo, G., Giedroc, D., Rajashekhar, B., & Villafranca, J. J. (1983d) *J. Biol. Chem. 259*, 1601–1606.

Friedman, S., & Kaufman, S. (1966) *J. Biol. Chem. 241*, 2256–2259.

Goldstein, M., Lauber, E., & McKereghan, M. R. (1965) *J. Biol. Chem. 240*, 2066–2072.

House, H. O., Chu, C. O., Wilkins, J. M., & Umen, M. J. (1975) *J. Org. Chem. 40*, 460–469.

Kaufman, S., & Friedman, S. (1965) *Pharmacol. Rev. 17*, 71–100.

Klinman, J. P., & Krueger, M. (1982) *Biochemistry 21*, 67–75.

Ljones, T., Skotland, T., & Flatmark, T. (1976) *Eur. J. Biochem. 61*, 525–533.

Lovenberg, W., Goodwin, J. S., & Wallace, E. F. (1975) *Neurobiological Mechanisms of Adaptation and Behavior* (Mandell, A. J., Ed.) p 77, Raven Press, New York.

May, S. W., Phillips, R. S., Mueller, P. W., & Herman, H. H. (1981) *J. Biol. Chem. 256*, 2258–2261.

May, S. W., Mueller, P. W., Padgette, S. R., Herman, H. H., & Phillips, R. S. (1983) *Biochem. Biophys. Res. Commun. 110*, 161–168.

Molinoff, P. B., & Axelrod, J. (1971) *Annu. Rev. Biochem. 40*, 465–500.

Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.

Rajashekhar, B., Fitzpatrick, P. F., Colombo, G., & Villafranca, J. J. (1984) *J. Biol. Chem. 259*, 6925–6930.

Rando, R. R. (1974) *Science (Washington, D.C.) 185*, 320–324.

Rando, R. R. (1977) *Methods Enzymol. 46*, 28–41.

Rosenberg, R. C., & Lovenberg, W. (1980) *Essays Neurochem. Neuropharmacol. 4*, 163–209.

Segel, I. H. (1975) *Enzyme Kinetics*, p 176, Wiley, New York.

Skotland, T., & Ljones, T. (1977) *Int. J. Pept. Protein Res. 10*, 311-314.
Skotland, T., & Ljones, T. (1979a) *Inorg. Perspect. Biol. Med. 2*, 151-180.
Skotland, T., & Ljones, T. (1979b) *Eur. J. Biochem. 94*, 145-151.
Taylor, K. B. (1974) *J. Biol. Chem. 249*, 454-458.
Villafranca, J. J. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 265-289, Wiley-Interscience, New York.
Walker, G. A., Kon, H., & Lovenberg, W. (1977) *Biochim. Biophys. Acta 482*, 309-322.
Wallace, E. F., Krantz, M. J., & Lovenberg, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2253-2255.
Walsh, C. (1982) *Tetrahedron 38*, 871-909.

# Active Site Directed *N*-Carboxymethyl Peptide Inhibitors of a Soluble Metalloendopeptidase from Rat Brain†

Thomas G. Chu and Marian Orlowski*

ABSTRACT: A soluble metalloendopeptidase isolated from rat brain preferentially cleaves bonds in peptides having aromatic residues in the $P_1$ and $P_2$ position. An additional aromatic residue in the $P_3'$ position greatly increases the binding affinity of the substrate, suggesting the presence of an extended substrate recognition site in the enzyme, capable of binding a minimum of five amino acid residues [Orlowski, M., Michaud, C., & Chu, T. G. (1983) *Eur. J. Biochem. 135*, 81-88]. A series of *N*-carboxymethyl peptide derivatives structurally related to model substrates and containing a carboxylate group capable of coordinating with the active site zinc atom were synthesized and tested as potential inhibitors. One of these inhibitors, *N*-[1(*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*-aminobenzoate, was found to be a potent competitive inhibitor of the enzyme with a $K_i$ of 1.94 $\mu$M. The two diastereomers of this inhibitor were separated by high-pressure liquid chromatography. The more potent diastereomer had a $K_i$ of 0.81 $\mu$M. The inhibitory potency of the less active diastereomer was lower by 1 order of magnitude. Decreasing the hydrophobicity of the residue binding the $S_1$ subsite of the enzyme by, for example, replacement of the phenylethyl group with a methyl residue decreased the inhibitory potency by almost 2 orders of magnitude. Deletion of the carboxylate group decreased the inhibitory potency by more than 3 orders of magnitude. Shortening the inhibitor chain by a single alanine residue had a similar effect. Binding of the inhibitor to the enzyme increased its thermal stability. The present data together with previous studies with synthetic and natural peptides support the conclusion that the active site of the enzyme contains two hydrophobic pockets at the $S_1$ and $S_3'$ subsites. As with other metalloendopeptidases [Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216-6220], effective inhibition requires the presence in the inhibitor of a group binding to the substrate recognition site of the enzyme and a group capable of coordinating with the active site metal atom.

Metalloproteases are a distinct class of proteolytic enzymes in which a zinc atom in the active site participates in the catalytic process. Some of these enzymes are credited with important physiological functions. For example, angiotensin converting enzyme (EC 3.4.15.1), a peptidyldipeptide hydrolase, is responsible for the conversion of angiotensin I to angiotensin II, thereby helping to regulate blood pressure. It has been suggested that the degradation of the endogenous opiate-like peptides Met- and Leu-enkephalin is controlled by a membrane-bound neutral metalloenzyme (Sullivan et al., 1978; Malfroy et al., 1978) later shown to be identical with a metalloendopeptidase isolated from bovine pituitaries (Orlowski & Wilk, 1981; Almenoff et al., 1981) and also rabbit kidney (Kerr & Kenny, 1974; Almenoff & Orlowski, 1983).

Metalloproteases are inhibited by simple anions and chelating agents, such as EDTA and 1,10-phenanthroline. Data have been presented showing that metal coordinating substrate analogues, compounds that incorporate characteristics essential for binding to the substrate recognition site of the enzyme and having a group capable of coordinating with the active site zinc atom, act as highly specific inhibitors of such enzymes (Holmquist & Vallee, 1979). Indeed, peptides fulfilling the binding requirements of thermolysin, a bacterial metalloendopeptidase, and containing *N*-terminal hydroxamate, thiol, carboxyl, or phosphoramidate zinc-coordinating groups have been shown to act as potent competitive inhibitors of this enzyme (Kam et al., 1979; Nishino & Powers, 1978). Similar highly specific inhibitors of angiotensin converting enzyme (Cushman et al., 1977; Patchett et al., 1980) and of membrane-bound metalloendopeptidase have also been synthesized (Roques et al., 1980; Mumford et al., 1982; Almenoff & Orlowski, 1983).

We have recently reported on the isolation from the soluble protein fraction of rat brain homogenates of a metalloendopeptidase ($M_r$ 67 000) optimally active at a neutral pH (Orlowski et al., 1983). Specificity studies indicate the presence of an extended substrate binding site with a preference toward peptides having aromatic or basic residues in the $P_1$ position,[1] or both the $P_1$ and $P_2$ positions, and an aromatic residue in the $P_3'$ position. Several natural peptides including bradykinin, neurotensin, substance P, and LHRH were hydrolyzed at sites

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[1] The nomenclature proposed by Schechter & Berger (1967) is used to define the position (P) of amino acids in the peptides and to name the subsites (S) in the active site of the enzyme.

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