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DOPAMINE-B-HYDROXYLASE: SUICIDE INHIBITION BY THE NOVEL OLEFINIC SUBSTRATE, 1-PHENYL-1-AMINOMETHYLETHENE

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SUMMARY: Dopamine-B-hydroxylase [E.C.1.14.17.1] plays a key role in the biosynthetic interconversion of neurotransmitters. It is now demonstrated for the first time that dopamine-B-hydroxylase also catalyzes the oxygenation of an olefinic substrate, 1-phenyl-1-aminomethylethene, producing 2,3-dihydroxy-2-phenylpropylamine after acid workup. This reaction gives the normal oxygenase stoichiometry of electrons to O_2 to product of 2:1:1, and is kinetically comparable to other oxygenase activities of dopamine-B-hydroxylase, with a k_{cat} value of 10 sec^{-1} and a K_M of 8.3 mM. 1-Phenyl-1-aminomethylethene is also a time-dependent, first-order inactivator of dopamine-B-hydroxylase. The inactivation process exhibits the characteristics of mechanism-based, irreversible inactivation, giving a K_I value of 13 mM and a k_{inac} of 0.04 min^{-1}. The central role of dopamine-B-hydroxylase in catecholamine metabolism suggests possible pharmacological uses for olefinic inhibitors of this enzyme.

INTRODUCTION: Dopamine-B-hydroxylase (DBH) [E.C. 1.14.17.1], a copper-containing monooxygenase present in a variety of mammalian tissues (1,2), catalyzes the conversion of dopamine to norepinephrine, thus playing a key role in the biosynthetic interconversion of neurotransmitters and in the production of adrenaline (3-6). The methylene hydroxylase activity of this enzyme has long been recognized, with a variety of substituted 2-phenethylamines serving as substrates (4). Previous work from this laboratory demonstrated for the first time two new oxygenase activities for DBH -- stereoselective sulfoxidation of aminoalkylphenyl sulfides and oxygenative ketonization of enantiomers of hydroxylation products through formation of the intermediate gem-diols (7-11). Both of these activities exhibit kinetic parameters comparable to those for

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Abbreviations: DBH, dopamine-B-hydroxylase; HPLC, high performance liquid chromatography; SNPA, N-succinimidyl-p-nitrophenylacetate; PAME, 1-phenyl-1-aminomethylethene; DHPPA, 2,3-dihydroxy-2-phenyl-1-propylamine; MES, 2-(N-morpholino)-ethanesulfonic acid.

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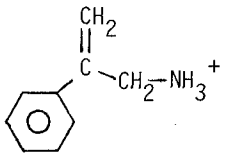
methylene hydroxylation, and the stereochemistry of all three processes is consistent. Thus, DBH, traditionally viewed as a specific "hydroxylase", exhibits considerable tolerance with respect to the functionality undergoing oxygenation. However, in a systematic study, we have found that it is considerably more selective than the broad-specificity microsomal P-450 and flavin monooxygenases (10).

In view of these findings, we have been interested in developing olefinic substrates for DBH with the expectation that DBH-catalyzed epoxidation of such substrates could well lead to suicide inactivation of the enzyme. Epoxidation by a purified "hydroxylase" was first demonstrated in this laboratory with the "w-hydroxylase" of Pseudomonas oleovorans (12-15). Ortiz de Montellano and coworkers have studied olefinic substrates for the highly non-specific P-450 oxygenation system, and have demonstrated that suicide inactivation occurs during epoxidation, with a radical or cationic intermediate likely leading to both epoxide formation and heme alkylations (16). Thus, there is precedence for the notion that properly designed olefins could be substrates for DBH which might lead to mechanism-based inactivation, but heretofore, epoxidative activity for this enzyme has never been demonstrated. We now wish to report the first example of an olefinic DBH substrate, and to demonstrate that time-dependent inactivation of DBH indeed occurs during the course of the enzymatic oxygenation of this substrate.

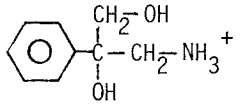
EXPERIMENTAL SECTION: Dopamine-B-hydroxylase was isolated from bovine adrenals as previously described (7-11) and exhibited a specific activity of 12-15 units/mg (ascorbate-supported). The enzymatic reaction was followed using either the polarographic oxygen-monitor assay or the spectrophotometric assay as previously described (9,10). HPLC analyses were performed using a Laboratory Data Control C-18 reverse phase column (10 cm, 5 A pore, low load) as previously described (9-11). Tyramine hydrochloride, ascorbic acid, and sodium fumarate were from Sigma, N-succinimidyl-p-nitrophenylacetate (SNPA) was from Pierce Chemical Company, and all other materials were from standard commercial sources and were of the highest grade obtainable. 1-Phenyl-1-aminomethylethene (PAME, I), the prototype olefinic substrate, was synthesized from alpha-bromomethyl styrene by either the method of Panzik and Mulvaney (17) or that of Patanova et al. (18), crystallized as the hydrochloride from EtOH/Et_2O, and characterized by NMR (TFA; q, 4.40 δ, 2H; S, 5.62 δ, 1H; S, 5.76 δ, 1H; S, 7.46 δ, 5H), mass spec (m/e 133, molecular ion), IR, and elemental analysis [mp 178-179°C.Calcd. for $C_9H_{12}NCl$: C, 63.71; H, 7.13; N, 8.26. Found: C, 63.78; H, 7.18; N, 8.25]. The diol, 2,3-dihydroxy -2-phenyl-1-propylamine hydrochloride (DHPPA, II) was synthesized by osmium tetroxide oxidation (19) of the N-triflouroacetyl derivitive of PAME, deblocked with NaOH, crystallized as the hydrochloride, and characterized by NMR (D_2O, trimethylsilyl propionic acid Na salt as internal

standard; S, 3.50 δ, 2H; S, 3.90 δ, 2H; S, 7.53 δ, 5H), mass spec (m/e 168, M + 1, chemical ionization), and elemental analysis [mp 152-153°C.Calcd. for $C_9H_{14}NO_2Cl$: C, 53.08; H, 6.93; N, 6.88. Found: C, 53.22; H, 6.93; N, 6.87].

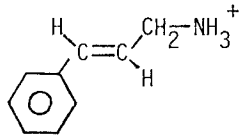
RESULTS AND DISCUSSION: Our initial efforts with olefinic suicide substrates focused on cinnamylamine (III), the olefinic analog of the straight-chain, saturated phenylalkylamine substrates.



I: PAME



II: DHPPA



III: Cinnamylamine

However, III proved to be a very poor DBH substrate, though indications of time-dependent inactivation were observed. Since it seemed probable that the side chain configuration imposed by the double bond in III was interfering with optimal orientation at the active site, we next turned our attention to PAME (I) as a prototypic olefinic substrate for DBH. Incubation of PAME with highly purified DBH under standard reaction conditions (9,10) in the presence of either $Fe(CN)_6^{4-}$ or ascorbate as electron donor, results in an enzyme-dependent consumption of both electrons and oxygen in the stoichiometry diagnostic for monooxygenase-catalyzed oxygenations, suggesting that enzymatic epoxidation of PAME is occuring. Kinetic constants obtained for both ascorbate- and ferrocyanide- supported oxygenation of PAME are presented in Table I along with comparative values obtained for other DBH substrates. It is apparent from the data that the reaction of DBH with PAME is kinetically comparable to DBH-catalyzed hydroxylation (2-phenethylamine, 3-phenylpropylamine), sulfoxidation (phenyl-2-aminoethyl sulfide), and ketonization (S-octopamine) reactions.

In order to establish that enzymatic oxygenation of PAME is indeed occurring, it was necessary to confirm the structure of the reaction product. However, it is clear that PAME-epoxide represents a highly unstable species, with aziridine formation, hydrolysis, and nucleophilic attack being among the processes which could occur readily. Thus, acid-trapping of the enzymatic product as its diol-hydrolysis product, DHPPA (II), was carried out.

TABLE I. KINETIC PARAMETERS OF DβH OXYGENATION REACTIONS

Substrate	Electron Donor: Ascorbic Acid[b] k_{cat}, S^{-1}	Km, mM	$k_{cat}/_{Km}$, $M^{-1}S^{-1}$	Electron Donor: $Fe(CN)_6^{4-}$[a] k_{cat}, S^{-1}	Km, mM	$k_{cat}/_{Km}$, $M^{-1}S^{-1}$
PAME	10	8.3	1.3×10^3	1	4.6	2.2×10^2
3-Phenylpropylamine	12	20.4	6.0×10^2	1	0.5	2.0×10^3
2-Phenylethylamine	65	7.0	9.0×10^3	19	1.0	1.9×10^4
Phenyl-2-aminoethyl sulfide	68	26.5	3.0×10^3	6	1.5	4.0×10^3
S-Octopamine	33	14.0	2.4×10^3	2.8	4.4	6.4×10^2

[a] The reaction system contains 5μM $CuSO_4$, 10mM sodium fumarate, and 1.5-2.0 mM $K_4Fe(CN)_6$ in 0.1 MES buffer, pH 6.0-6.1, at varying amount of substrate. The reaction was followed spectrophotometrically at 420 nm ($\Delta\varepsilon$=1100 $M^{-1}cm^{-1}$) and the cell compartment was thermostatted at 37°C.

[b] The reaction system contains 5μM $CuSO_4$, 8-10 mM sodium fumarate, 10-12 mM ascorbic acid, 200-300 μg/ml catalase, and varying amounts of substrate, in 0.1M acetate buffer, pH 5.0. The reaction was followed by measuring O_2 uptake at 37°C with a Clark polarographic electrode and a YSI Model 53 O_2 monitor. Details of the assay method have been published elsewhere (9,10).

TABLE II. COMPARATIVE STOICHIOMETRY FOR DBH-CATALYZED OXYGENATION REACTIONS

Oxygenated Substrate	$\frac{[Fe(CN)_6^{4-}]}{[O_2]}$	$\frac{[O_2]}{[Product]}$
PAME (I)	1.9[a,b,c]	1.1[d,e]
Tyramine	2.1[f]	-[g]
Phenyl-2-Aminoethylsulfide	2.3[f]	0.8[f]

a. Determined as in footnote a, Table I.
b. Determined as in footnote b, Table I.
c. Average of two determinations.
d. O_2 uptake determined as in footnote b, Table I, except that 0.13 mg purified DBH was used, at an initial PAME concentration of 20 mM. The reaction was quenched with 170 µL 9M H_2SO_4 (to pH 0.3) and incubated with shaking for 2 hours. After centrifugation, internal standard addition (0.5 µ moles phenethylamine HCl), basification, and extraction with ethyl acetate, the diol was analyzed as the SNPA derivitive by HPLC using the standard procedure as previously described (22). Using a standard curve based on peak area diol/peak area standard, enzymatic-derived diol was determined by subtraction of diol found in a control (no DBH) from that found in the enzymatic reaction.
e. Average of five determinations.
f. Calculated from reference (7).
g. The stoichiometry of product formed per 2 equiv. of $Fe(CN)_6^{4-}$ has been reported as 1:1 (23).

Quantitative HPLC procedures for isolation and identification of DHPPA were developed in order to allow comparison with synthetic DHPPA and determination of the stoichiometry of product formation. Following incubation of PAME with DBH, the reaction was quenched with H_2SO_4 and, after basification and extraction, the diol (II) was detected by HPLC as the N-p-nitrophenylacetate derivative using phenethylamine as an internal standard. Control experiments using synthetic DHPPA established that the amount of diol present in the reaction mixture can be quantitatively determined using this procedure. The analysis of DHPPA (II) was complicated by the formation of small amounts of non-enzymatic diol in control reactions not containing DBH. This non-enzymatic diol component, shown to be formed during the H_2SO_4 hydrolysis step in the presence of ascorbate, was subtracted from the total diol detected in the enzymatic reaction to give the DHPPA (II) due to enzymatic oxygenation. Table II summarizes comparative stoichiometry data obtained for PAME and other known DBH substrates. It is apparent that the expected electrons to oxygen to product stoichiometry of 2:1:1 is obtained, indicating that PAME reacts via the normal oxygenative pathway.

Examination of the time course of the reaction of PAME with DBH revealed that the enzyme undergoes a time-dependent inactivation during the course of the

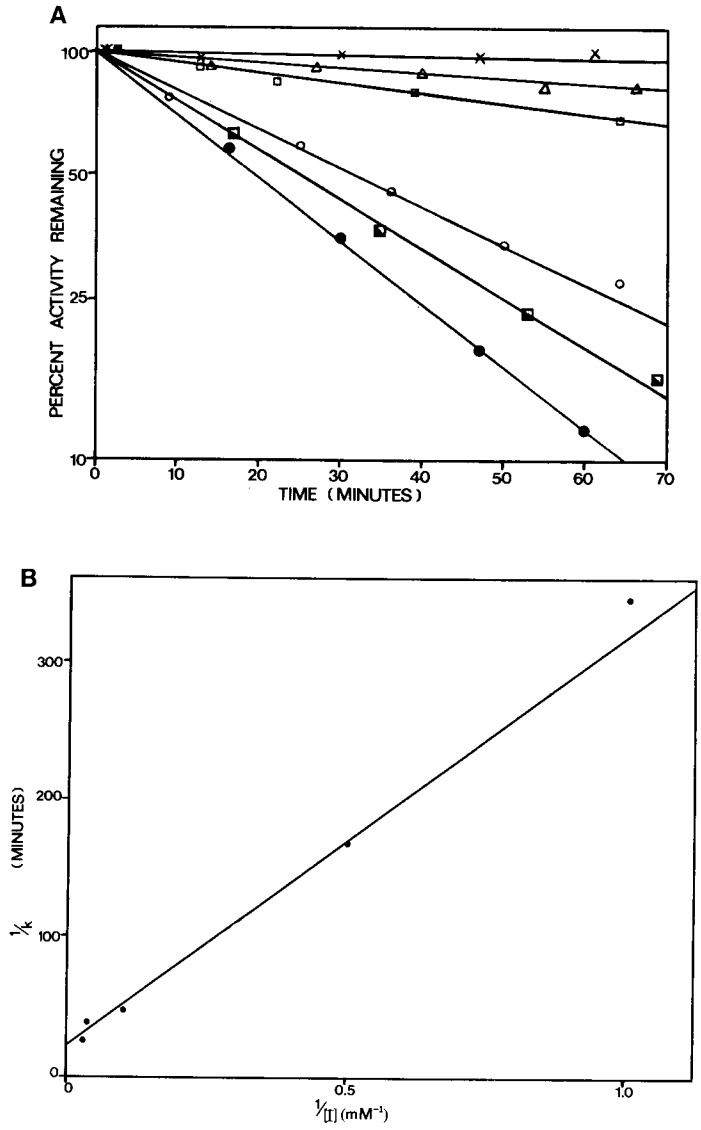


FIGURE I. (A) TIME-DEPENDENT INACTIVATION OF DBH BY PAME. The enzyme inactivation mixtures contained 0.10 M sodium acetate buffer, pH 5.0, 9-10 mM sodium fumarate, 5.5-6.0 μM $CuSO_4$, 1.1-1.3 mg/ml catalase, and 0.05 mg/ml DBH, at various PAME concentrations in a 0.40 ml total volume. The inactivation reactions, performed at room temperature, were initiated by adding an appropriate amount of ascorbate to bring the ascorbate concentration to 5.0 mM. The activity of DBH versus time was assayed at 37.0°C by the dilution of 25 μL aliquots of the inactivation mixture into assay solutions identical to those described in Table I, b., except that 10 mM tyramine was included in a total volume of 2.50 ml. Lines resulting from plots of ln(percent activity remaining) vs. time were fitted by linear regression, and first-order rate constants were calculated as the slopes of these lines. X, control (no PAME); △, 1.0 mM PAME; □, 2.0 mM PAME; ○, 10 mM PAME; ◪, 30 mM PAME; ●, 40 mM PAME.
(B) A plot of the above data as $1/k_{obs}$ vs. $1/[I]$ gives the expected linear relationship. The values of K_I and k_{inac} were determined by using the computer program of Cleland (24) to fit the data to the hyperbolic form of the inactivation expression $k_{obs} = [I]k_{inac} /([I] + K_I)$.

oxygenation reaction. As illustrated in Figure I, inactivation is strictly first-order and its rate is dependent on PAME concentration, as would be expected for suicide inactivation by a reactive species generated within the active site. The double-reciprocal plot shown in Fig. 1B yields the values K_I = 13 mM and k_{inac} = 0.04 min^{-1}, assuming the simplest kinetic scheme for suicide inactivation (20). Inclusion of the substrate, tyramine, in the incubation mixture protects against inactivation by PAME, and the inactivation process is also dependent on the presence of a reducing agent- - either ascorbate or ferrocyanide - - in the reaction mixture. Furthermore, inactivation of DBH by PAME goes completely to 100% inactivation and no reappearance of enzyme activity is evident even after prolonged incubation in the presence of acetate. Similarly, no reversal of inactivation is observed after prolonged dialysis. The latter two points are particularly significant in view of the recent report of Columbo, et al. that DBH inhibition by benzylcyanide can be reversed (21).

Taken together, the data presented here establish for the first time that DBH readily carries out the oxygenation of an olefinic substrate. This finding further extends the specificity of an enzyme previously considered to be a specific "hydroxylase", but which we have shown to be capable of carrying out sulfoxidation and ketonization reactions (7-11). The chemical nature of the species responsible for suicide inactivation is not yet clear, and it may be either the epoxide or an intermediate formed along the pathway of olefin oxygenation, as has been proposed for P-450 inactivation (16). In view of the central role of DBH in catecholamine metabolism, the demonstration of suicide inhibition by an olefinic substrate may establish the basis for the design of a new class of DBH inhibitors of pharmacological importance.

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