Deuterium Isotope Effect Studies on the MAO-B Catalyzed Oxidation of 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine†

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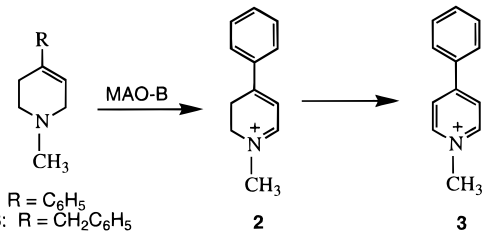
ABSTRACT: Previous studies have established that 1-cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine is an efficient time- and concentration-dependent inhibitor of the flavin-containing enzyme monoamine oxidase B (MAO-B). This behavior is consistent with a proposed mechanism-based inactivation pathway which proceeds via an initial single electron transfer step to generate an unstable cyclopropylaminyl radical cation intermediate that alkylates an active site functionality via the ring opened primary carbon centered radical. More recently we have found that, in addition to being an inhibitor, the corresponding 1-cyclopropyl-4-benzyl-1,2,3,6-tetrahydropyridine species is an excellent MAO-B substrate, behavior which may not be consistent with the obligatory formation of a cyclopropylaminyl radical cation intermediate. In an attempt to gain further insight into the mechanism associated with the MAO catalyzed oxidation of 1,4-disubstituted tetrahydropyridines, we have undertaken deuterium isotope effect studies on the substrate and inhibitor properties of this 4-benzyl-1-cyclopropyltetrahydropyridine derivative. A normal isotope effect was observed on k_{cat}/K_M. Although the good substrate properties of this compound prevented an accurate estimate of k_{inact} and K_I, we did observe a very modest inverse isotope effect on the rate of inactivation of 0.1 μM MAO-B by 500 μM inactivator. The results are discussed in terms of possible mechanisms for the MAO-B catalyzed oxidation of 1,4-disubstituted 1,2,3,6-tetrahyrdopyridines.

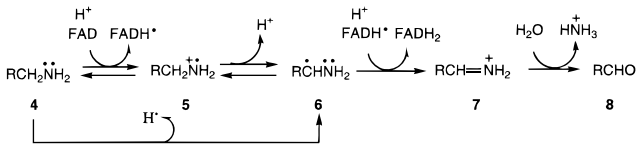
The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP,[1] **1**) causes the selective destruction of nigrostriatal neurons leading to a Parkinsonian syndrome in humans has stimulated interest in delineating the mechanism of action of this neurotoxin (Langston, 1989). Previous studies have established that the neurotoxicity of MPTP is mediated by the corresponding pyridinium species MPP^+ (**3**) which is generated via the dihydropyridinium intermediate $MPDP^+$ (**2**) in a reaction sequence catalyzed by monoamine oxidase B (MAO-B) and, less efficiently, by MAO-A as illustrated in Scheme 1 (Chiba et al., 1984; Salach et al., 1984). The excellent MAO-B substrate properties of MPTP were unexpected since previously no cyclic tertiary amines had been shown to be substrates for this enzyme. These characteristics of MPTP have prompted us (Kalgutkar et al., 1994; Zhao et al., 1992; Kalgutkar & Castagnoli, 1992) and others (Efange et al., 1993; Krueger et al., 1992; Youngster et al., 1989) to explore the special structural features of 1,4-disubstituted 1,2,3,6-tetrahydropyridines that are responsible for their unique MAO substrate properties.

One proposed mechanism for the MAO-catalyzed α-carbon oxidative deamination of amines that has been studied extensively proceeds by an initial single electron transfer (SET) step from the nitrogen lone pair of the substrate (**4**)

Scheme 1. MAO-B Catalyzed Oxidation of MPTP (**1**)



Scheme 2. Proposed Pathways for the MAO-Catalyzed Oxidation of Amines



to the oxidized flavin (FAD) to generate an aminyl radical cation (**5**) and the flavin radical $FADH^•$ (Scheme 2). α-Carbon deprotonation of **5** yields the carbon-centered radical **6** which undergoes a second one-electron oxidation to form the iminium product **7** and the fully reduced flavin ($FADH_2$). Subsequent hydrolysis of **7** provides the deaminated product **8** (Silverman, 1991, 1995b). This SET mechanism is supported by results from studies on mechanism-based MAO-B inactivators such as *N*-benzylcyclopropylamine (**9**) and model photochemical (Kim et al., 1991) and ESR (Yelekci et al., 1989) studies. The putative cyclopropylaminyl radical cation **10** generated by the SET pathway (Scheme 3) is proposed to undergo rapid ring opening to form the highly reactive primary radical **11** which mediates the inactivation of the enzyme (Silverman & Yamasaki, 1984; Silverman & Zieske, 1985, 1986). Ad-

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[1] Abbreviations: MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SET, single electron transfer; FAD, flavin adenine dinucleotide; GC/EIMS, gas chromatography−electron ionization mass spectrometry; NMR, nuclear magnetic resonance.

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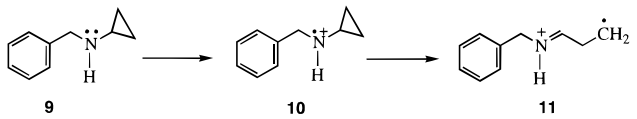
Scheme 3. Proposed MAO-B Catalyzed SET Pathway for *N*-Benzylcyclopropylamine (**9**) Leading to the Primary Carbon Radical **11**



9 **10** **11**

ditional evidence considered to be consistent with this mechanism is the similarity observed in the isotope effects on k_{cat}/K_M and k_{inact}/K_I for milacemide (Silverman et al., 1993).

We have found that 1-cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine (**12**) is a good time- and concentration-dependent inhibitor of MAO-B (Hall et al., 1992). This behavior is consistent with a radical reaction pathway (Scheme 4) analogous to that proposed by Silverman for *N*-benzylcyclopropylamine (Scheme 2). Initial one-electron transfer from **12** to FAD would generate the cyclopropylaminyl radical cation **13**. This intermediate would have the option to ring open to the reactive primary radical **14** (pathway **a** leading to enzyme inactivation) or to lose an α-proton (pathway **b**) to yield the resonance stabilized allylic radical **15a** ↔ **15b** which subsequently would undergo a second one-electron loss to generate the dihydropyridinium intermediate **16**. Since the dihydropyridinium metabolite was not detected under conditions leading to enzyme inactivation, one may conclude that the rate of ring opening must be much faster than the rate of α-proton loss as would be anticipated by the very rapid ring opening rates reported for cyclopropylcarbinyl (Atkinson & Ingold, 1993; Martin-Esker et al., 1994) and cyclopropylaminyl (Maeda & Ingold, 1980) radicals.

More recently, we examined the interactions of 4-benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine (**17**) with MAO-B (Kuttab et al., 1994). The corresponding *N*-methyl analog **18** (see Scheme 1) is a better MAO-B substrate than is MPTP (Naiman et al., 1990), and therefore we anticipated that **17** would display more potent inactivator properties. Although **17** proved to be a time- and concentration-dependent inhibitor of MAO-B, it unexpectedly displayed excellent MAO-B substrate properties. According to the SET mechanism, the intermediate cyclopropylaminyl radical cation **19** must partition between pathway **a**, leading to the primary carbon centered radical **20** and enzyme inactivation, and pathway **b**, leading to the allylic radical intermediate **21a** ↔ **21b** and product (**22**) formation (Scheme 4). Rough estimates of the partition ratio between these two pathways suggested that over 1000 substrate molecules should be converted to product for each molecule of enzyme that undergoes inactivation. These considerations argue either that opening of the cyclopropyl ring of the radical cation **19** is slower than proton loss at C-6 or that formation of **22** does not proceed via **19**.

In an attempt to gain additional insight into the interactions of **17** with MAO-B, we have examined the deuterium isotope effects on the rates of substrate turnover and enzyme inhibition. The studies were carried out with 4-benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d_4 (**17**-d_4) and a purified form of MAO-B isolated from beef liver.

EXPERIMENTAL PROCEDURES

Chemistry. CAUTION: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**1**) is a known nigrostriatal neurotoxin and should be handled using disposable gloves in a properly ventilated hood. Detailed procedures for the safe handling of MPTP have been reported (Pitts et al., 1986).

Synthetic reactions were carried out under a nitrogen atmosphere. *R*-(−)- deprenyl was obtained from Research Biochemicals Inc., Natic, MA. All other chemicals (Aldrich, Milwaukee, WI) were reagent or HPLC grade. Proton NMR spectra were recorded on a Bruker WP 270-MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane as internal standard. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), or m (multiplet). Gas chromatography/electron ionization mass spectrometry (GC/EIMS) was performed under electron ionization conditions using a Hewlett Packard (HP) 5890 capillary GC equipped with an HP-1 capillary column (12-m × 200-mm × 0.33-mm film thickness) coupled to a HP 5970 mass-selective detector. Data were acquired using a HP 5970 MS ChemStation. Normalized peak heights are reported as percentage of the base peak. Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected.

*Methyl Acrylate-2,2-d_2 (**24**).* A mixture of anhydrous DMSO (60 mL) and NaH (2.32 g of 60% oil dispersion, 58 mmol) was stirred under N_2 at 65 °C until a clear solution resulted. To this solution was added portionwise (1 h) with stirring at room temperature 24 g (58 mmol) of methoxycarbonylmethyltriphenylphosphonium bromide [**23**, mp 167−168 °C; lit. (Ayrey & Wong, 1978) mp 162−163 °C] to generate the Wittig reagent. Solid paraformaldehyde-d_2 (2 g, 63 mmol) was cracked in a separate flask at 200 °C, and the resulting vapors were condensed in the reaction flask, maintained at 90 °C, that contained the above Wittig reagent. The reaction mixture was stirred and maintained at 90 °C for 2 h and then stirred overnight at room temperature. The product (2.66 g, 53%) was obtained by vacuum distillation (50 torr) with the receiver cooled to −78 °C: ^{1}H NMR ($CDCl_3$) δ 6.12 (s, CH, 1H), 3.76 (s, OCH_3, 3H).

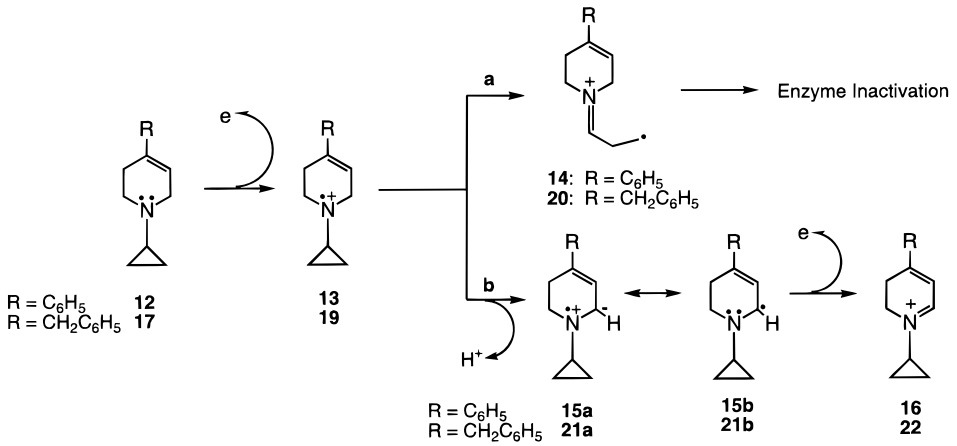
*Oxalate Salt of 1-Cyclopropyl-4-benzyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d_4 (**17**-d_4).* Synthesis of the desired 2,2,6,6-tetradeutero analog **17**-d_4 was achieved by following the literature procedure for the d_0-compound **17**-d_0 (Kuttab et al., 1994). The physicochemical characteristics of the final product and the intermediate species are summarized below.

N,N-Bismethoxycarbonylmethylcyclopropylamine-d_4. Yellow oil, (89%); bp 114−115 °C/50 Torr; ^{1}H NMR ($CDCl_3$) δ 3.66 (s, OCH_3, 6H), 2.50 (s, NCD_2CH_2, 4H), 2.15 (m, $NCHCH_2$, 1H), 0.40 (m, $NCHCH_2$, 4H); GC/EIMS (temperature program, 125 °C for 1 min followed by a ramp of 10 °C/min for 10 min) showed a single peak with t_R = 3.17 min and *m/z* 233 ($M^{\bullet+}$, 12), 174 (30), 160 (100), 89 (34), 59 (48).

1-Cyclopropyl-4-piperidone-2,2,6,6-d_4. Yellow oil (51%); ^{1}H NMR ($CDCl_3$) δ 2.41 (s, NCD_2CH_2, 4H), 1.78 (m, $NCHCH_2$, 1H), 0.53 (m, $NCHCH_2$, 4H); GC/EIMS (temperature program, 80 °C for 2 min followed by a ramp of 10 °C/min for 15 min) showed a single peak with t_R = 4.44 min and *m/z* 143 ($M^{\bullet+}$, 20), 100 (40), 99 (80), 85 (62), 70 (100), 55 (78).

4-Benzyl-1-cyclopropyl-4-piperidinol-d_4. Yellow oil (77%); GC/EIMS (temperature program, 125 °C for 1 min followed by a ramp of 10 °C/min for 10 min) showed a single peak with t_R = 4.774 min and *m/z* 235 ($M^{\bullet+}$, 38), 205 (42), 144 (40), 101 (65), 85 (100), 72 (78).

Scheme 4. Proposed SET Pathway for the MAO-B Catalyzed Oxidation of 1-Cyclopropyltetrahydropyridine Derivatives **12** and **17**



*Oxalate Salt of 1-Cyclopropyl-4-benzyl-1,2,3,6-tetrahydropyridine-d_4 (**17**-d_4).* Mp 144−145 °C [**17**-d_0 lit. (Kuttab et al., 1994) mp 148−149 °C]; ^{1}H NMR (DMSO-d_6) δ 7.1−7.4 (m, ArH, 5H), 5.45 (bs, NCD_2CH, 1H), 3.31 (s, $ArCH_2$, 2H), 2.49 (m, $NCHCH_2$, 1H), 2.10 (s, NCD_2CH_2, 2H), 0.6−0.8(m, $NCHCH_2$, 4H); GC/EIMS (temperature program, 125 °C for 1 min followed by a ramp of 10 °C/min for 8 min) showed a single peak with t_R = 4.14 min and *m/z* 217 ($M^{\bullet+}$, 65), 201 (100), 91 (55), 70 (40).

Enzymology. The isolation and purification of MAO-B from beef liver was carried out using the methodology reported earlier (Salach & Weyler, 1987) with the following variations. The phospholipase A used in our preparation was obtained commercially (Sigma, St. Louis, MO) rather than from the crude venom, and we did not subject the preparation to the sucrose gradient purification step. We obtained a highly active and stable preparation which was stored at −15 °C in 200−400 μL aliquots. A 0.09 μM solution of the enzyme was transparent in the UV range of interest. The specific activity was determined spectrophotometrically at 30 °C on a Beckman DU-50 spectrophotometer by estimating the initial (30−120 s) rates of conversion of 5 mM MPTP (**1**) to the dihydropyridinium species **2** (λ_{max} = 343 nm, ϵ =16 000 M^{-1}, k_{cat} = 204 min^{-1}) as described previously (Kalgutkar et al., 1994). The final enzyme concentration was calculated to be 9.0 nmol/mL.

All enzyme assays were performed in triplicate at 37 °C with a Beckman DU-50 spectrophotometer. The MAO-B substrate properties of **17**-d_0 and **17**-d_4 were examined following published procedures (Kalgutkar et al., 1994). The molar extinction coefficient of the corresponding dihydropyridinium species was taken to be 5530 M^{-1}, corresponding to 4-benzyl-1-methyl-2,3-dihydropyridinium perchlorate (Naiman et al., 1990).

The MAO-B inactivation properties of **17**-d_0 and **17**-d_4 were examined following published procedures (Rimoldi et al., 1995). A substrate protection experiment was performed with benzylamine and **17**-d_0. A mixture of MAO-B (0.8 μM), **17**-d_0 (0.4 mM), and benzylamine (2 mM) was gently agitated in a water bath incubated at 37 °C. Aliquots (25 μL) removed at 0, 4, 8, and 12 min were assayed for remaining enzyme activity using 5 mM MPTP (475 μL) as described previously (Hall et al., 1992).

*Determination of the Partition Ratio for **17**-d_0 and **17**-d_4.* A 0.5 mL mixture of MAO-B (0.09 μM) and **17**-d_0 or **17**-d_4 (0.5 mM) was allowed to incubate at 37 °C for 1 min during which time dihydropyridinium product **22**-d_0 or **22**-d_3 formation was recorded spectrophotometrically at 296 nm. Identical 1 min incubation mixtures were assayed for remaining enzyme activity by adding an equal volume of 10 mM MPTP and determining the rate of $MPDP^+$ formation as usually performed in our inactivation studies. The ratio of nmol of product formed per nmol of enzyme inactivated was calculated from these measurements, and the corresponding deuterium isotope effect on the partition ratio was obtained from the partition ratios for **17**-d_0 and **17**-d_4.

*GC/EIMS Assay for **17**-d_0.* A 500 μL mixture containing 0.5 mM **17**-d_0 and 4.5 μM MAO-B was incubated at 37 °C. Aliquots (20 μL) removed at 0, 2, 4, 6, and 8 min were added to 480 μL of 10% aqueous K_2CO_3 maintained at 0 °C. The resulting basic solution was extracted with ethyl acetate (1 mL) and the organic layer was dried over $MgSO_4$. A 1 μL sample was analyzed by GC-EIMS selected ion monitoring at *m/z* 213 (temperature program: 125 °C for 1 min followed by a ramp of 25 °C/min for 5 min and then a ramp of 50 °C/min to 300 °C). In a control experiment, MAO-B was preincubated with 10 μM deprenyl for 10 min at 37 °C and then treated as above.

*Determination of the Irreversibility of the Inactivation of MAO-B by **17**-d_0.* A 200 μL mixture containing 2.3 μM MAO-B and 0.5 mM **17**-d_0 in 100 mM sodium phosphate buffer pH 7.4 was incubated at 37 °C for 3 h. After adding 100 μL of 0.3% Blue Dextran solution in the same buffer the solution was applied to Sephadex G-25 (1.5 × 6 cm) previously equilibrated with 100 mM sodium phosphate buffer, pH 7.4, and eluted with the same buffer at a flow rate of 1.0 mL/min. The fraction containing the majority of the Blue Dextran was collected and assayed for enzyme activity using 5 mM MPTP. In a control experiment, 2.3 μM MAO-B was incubated with buffer for 3 h at 37 °C, and the procedure was repeated as above.

RESULTS

Chemistry. The synthesis of 4-benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d_4 (**17**-d_4) followed the reported synthesis of the d_0 compound (**17**-d_0) (Kuttab et al., 1994). The key deuterated starting material, methyl acrylate-d_2, was prepared via a Wittig reaction following a literature procedure for the synthesis of methyl methacrylate-d_2 (Ayrey & Wong, 1978).

Enzymology. The kinetic deuterium isotope effects on k_{cat} and K_M for the MAO-B catalyzed oxidation of **17**-d_0 and **17**-d_4 were estimated by measuring the rates of formation of the corresponding dihydropyridinium metabolites **22**-d_0 and **22**-d_3 spectrophotometrically over a substrate concentration range of 150−1000 μM in the presence of a fixed concentration (0.1 μM) of MAO-B. At the higher substrate concentrations the plots of time vs concentration of dihydropyridinium metabolites were curvilinear. Since only a small percentage of the substrate would have been consumed in these experiments during the 2 min data collection periods, the slowing down of the rate of product formation was assumed to be a consequence of the inactivation of the enzyme. Despite this problem, the double reciprocal plots generated from these rate data gave straight lines ($r^2 > 0.97$) for both the d_0 and the d_4 substrates. The k_{cat} and K_M values estimated for the d_0 substrate (810 ± 78 min^{-1} and 0.40 ± 0.08 mM, respectively) compared well with the corresponding values (637 min^{-1} and 0.40 mM) reported by us previously (Kuttab et al., 1994) The corresponding analyses for **17**-d_4 gave values of 530 ± 65 min^{-1} for k_{cat} and 0.37 ± 0.07 mM for K_M. Based on these data, the deuterium kinetic isotope effect on k_{cat}/K_M is 1.4 and on k_{cat} 1.5. This intermolecular isotope effect argues that cleavage of the allylic carbon−hydrogen bond is at least partially rate determining in the MAO-B catalyzed oxidation of **17** to the corresponding dihydropyridinium metabolite **22**.

The previously observed concentration- and time-dependent MAO-B inhibition properties of **17** were interpreted as being consistent with a mechanism-based inactivation pathway (Silverman, 1988). Additional evidence to support this tentative conclusion now has been obtained. As expected, the preferred MAO-B substrate benzylamine (2 mM) protected against loss of enzyme activity mediated by **17**-d_0 (500 μM). After an 8 min incubation period only 10% of the original activity remained in the absence of benzylamine while 68% remained in the presence of benzylamine. We also examined the reversibility of the inhibition. MAO-B (2.3 μM) was completely inhibited following incubation with **17**-d_0 (500 μM) for 180 min. The enzyme recovered following passage through Sephadex G-25 showed no recovery of activity. In a control experiment (buffer only) over 90% of the original activity was recovered. Consequently, it is reasonable to assume that **17** is a classical mechanism-based inactivator that inhibits MAO-B through covalent bond formation between an enzyme generated reactive species and an active site functionality.

The inactivation curve observed in the above experiment showed biphasic kinetic behavior with the initial, rapid phase having a $k_{obs} = 0.06$ min^{-1} and the second, slow phase with $k_{obs} = 0.005$ min^{-1}. Consequently, it appeared that product inhibition also was contributing to the loss of enzyme activity. This conclusion was confirmed by actual measurements of remaining substrate vs time of a typical incubation (500 μM **17**-d_0 and 4.5 μM MAO-B) used in our attempts to estimate k_{inact} and K_I (see below). Quantitative GCMS analysis of this incubation mixture showed that the remaining concentration of **17**-d_0 after a 2 min incubation period was only 2 μM, a substrate concentration which would show no detectable enzyme inhibition at these enzyme concentrations. On the other hand, 54 ± 8% (average of four experiments) of the enzyme activity was still present at this time. The remaining enzyme activity, however, continued to decline slowly, presumably via an enzyme generated metabolite, possibly the dihydropyridinium species **22**. Since no enzyme activity is recoverable following Sephadex chromatography, both types of inactivation must be irreversible. Although we have not been able to synthesize **22** to perform kinetic studies, inactivation properties have been observed with the MAO-B generated dihydropyridinium metabolite **2** derived from MPTP (Singer et al., 1986).

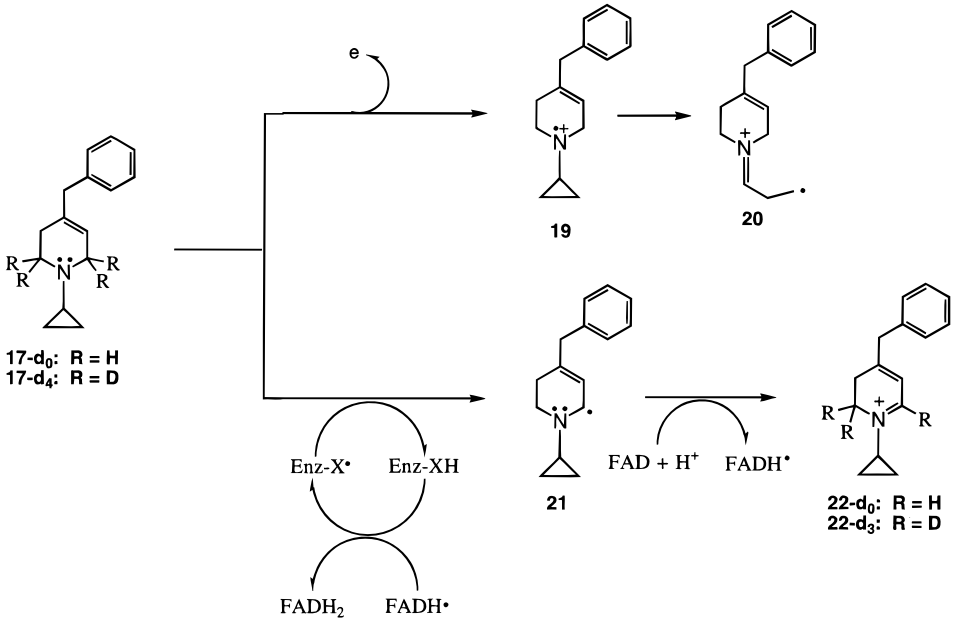
Previous attempts to estimate k_{inact} and K_I for the inactivation of 4.5 μM MAO-B by **17** in which the reaction mixture was sampled every 4 min displayed non-pseudo-first-order kinetics (Silverman, 1995a), presumably because of the high partition ratio and the associated rapid consumption of the inactivator. We have made an additional attempt to collect useful inactivation kinetic data by sampling the incubation mixture for remaining enzyme activity every 1 min. Even under these conditions, however, the kinetics of inactivation still did not follow Michaelis−Menton behavior, i.e., the double-reciprocal plot of k_{obs} vs inhibitor concentration gave an X-intercept greater than zero. Due to the limited solubility properties of **17**, we were not able to examine a greater concentration range which might have provided more reliable data.

In an effort to obtain a useful estimate of the influence of deuterium substitution on the relative rates at which **17**-d_0 and **17**-d_4 inactivate MAO-B, we determined the nanomoles of enzyme inactivated at 1 min in an incubation mixture containing 500 μM **17**-d_0 or **17**-d_4 and 0.1 μM MAO-B, that is, under incubation conditions in which less than 10% of the substrate would be consumed at the time of the measurement. The experiment was ran in triplicate and gave values of 32.0 ± 0.6 × 10^{-3} nmol of MAO-B inactivated/(mL·min) for **17**-d_0 and 35.0 ± 1.9 × 10^{-3} nmol of MAO-B inactivated/(mL·min) for **17**-d_4. From these data an isotope effect of 0.92 (0.85−0.98) was calculated for the rate of inactivation of 0.1 μM MAO-B by 500 μM **17**. The rates of substrate turnover under the same conditions gave values of 40.0 ± 2.5 nmol of dihydropyridinium formed/(mL·min) for **17**-d_0 and 28.0 ± 1.7 nmol of dihydropyridinium formed/(mL·min) for **17**-d_4. The corresponding partition ratios are 1250 (**17**-d_0) and 800 (**17**-d_4) nmol of dihydropyridinium formed/nmol of MAO-B inactivated, and the isotope effect on the partition ratio is 1.6.

DISCUSSION

Initial electron transfer vs hydrogen atom transfer in enzyme-catalyzed oxidations of amines has been a topic of debate. Although the SET pathway has enjoyed much favor, it also has been challenged. Results from rapid-scan stopped flow and magnetic field effect studies on the MAO-B catalyzed oxidation of [α,α-^{2}H]benzylamine have led Edmondson to argue against the SET pathway since he could find no evidence of the formation of a flavin radical pair intermediate in this reaction (Miller et al., 1995). Edmondson also has suggested that single-electron transfer from an amine to the flavin is thermodynamically and kinetically improbable since the energy barrier for electron transfer is greater than the energy barrier for the reduction of FAD to $FADH_2$ (Walker & Edmondson, 1994). A similar debate concerns the cytochrome P450 catalyzed oxidations of tertiary amines (Hanzlik & Tullman, 1982; Guengerich & Macdonald, 1984; Miwa et al., 1983; Karki et al., 1995;

Scheme 5. Proposed Pathway for the MAO-B Catalyzed Oxidation of 1-Cyclopropyltetrahydropyridine Derivatives



Carlson et al., 1995). Energy estimates derived from chemical models and the enzyme-catalyzed oxidation of *N*,*N*-dimethylaniline derivatives again suggest that, even in polar solvents that might stabilize the radical cation, hydrogen atom transfer is energetically preferred over the SET pathway by several kcal/mol (Dinnocenzo et al., 1993). Similar conclusions based on isotope effects and stereochemical arguments have been reached in the case of the cytochrome P450-catalyzed α-carbon oxidation of (*S*)-nicotine (Carlson et al., 1995).

The unexpected substrate properties of **17** have prompted us to consider the possibility that the cyclopropylaminyl radical cation also may not be an obligatory intermediate in the MAO-B catalytic pathway of these tetrahydropyridine derivatives. Opening of the cyclopropyl ring via a conformation which allows overlap of the half filled p-orbital of the radical cation with the p-like orbitals of the cyclopropyl carbon−carbon bonds is considered to be an energetically favored process because of the release of ring strain (Silverman, 1995b). Nevertheless, conformational constraints imposed by the active site on the conformation of the tetrahydropyridine substrate could restrict orbital alignments in which case an alternative reaction, proceeding through an active site base promoted deprotonation at the allylic C-6 position, might compete kinetically with the ring opening reaction. In this case, formation of the dihydropyridinium product could dominate the reaction as, in fact, is observed with the MAO-B catalyzed oxidation of **17**. On the other hand, one also could account for the observed substrate and inactivation properties of **17** if partitioning were to occur between an electron transfer pathway and a pathway proceeding via direct carbon−hydrogen bond cleavage.

Fitzpatrick and Villafranca (1986) have proposed such a partitioning for mixed substrate/inactivators of dopamine β-hydroxylase such as benzylhydrazine. These investigators report that the observed normal deuterium isotope effect on the partition ratio, the inverse deuterium isotope effect on k_{inact}/K_I, and the normal deuterium isotope effect on V_{max} are most consistent with a partitioning that occurs at the point of C−H bond cleavage and that this bond cleavage is involved only in product formation and not in inactivation.

Although we have not been able to obtain k_{inact} and K_I values, the results of our kinetic studies suggest a weak inverse isotope on the inactivation of MAO-B by **17** and a normal isotope effect on product formation and the partition ratio, behavior that is analogous to the kinetic pattern reported by Fitzpatrick and Villafranca. Consequently, although partitioning following electron transfer cannot be ruled out, we interpret the results reported here as consistent with a pathway involving the partitioning of **17** between the single-electron transfer product **19** and the hydrogen atom transfer product **21** (Scheme 5). Such a process presumably would involve the participation of a protein derived radical species (Enz-X•) that would generate the allylic radical intermediate **21**. One-electron oxidation of **21** by FAD would result in product **22** formation. Finally, the enzyme-bound radical would be regenerated in a reaction coupled to the reduction of FADH• to $FADH_2$. An inverse isotope effect on inactivation would be expected since the normal isotope effect on hydrogen atom abstraction would increase the probability of the one-electron oxidation of **17** to **19**. This pathway is attractive in that it obviates the need to invoke a kinetic preference for proton loss over ring opening of a cyclopropylaminyl radical cation and is consistent with the direct formation of a highly resonance stabilized (by the nitrogen lone pair and the styryl system) carbon radical.

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