**Deuterium Isotope Effect Measurements on the Interactions of the Neurotoxin 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine with Monoamine Oxidase B**

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Kinetic deuterium isotope effect studies for the noncompetitive, intermolecular monoamine oxidase B-catalyzed oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the corresponding 1-methyl-4-phenyl-2,3-dihydrodipyridinium species MPDP* were found to be 3.55 on \( V_{\text{max}} \) and 8.01 on \( V_{\text{max}}/K_m \) with MPTP-6,6-d2 as the deuterated substrate. Similar values were obtained with MPTP-2,2,6,6-d4 and MPTP-CD3-2,2,6,6-d4. The deuterium isotope effect for the electrochemical oxidation of 1 mM MPTP-2,2,6,6-d4 was only 1.35. These results indicate that the monoamine oxidase B-catalyzed oxidation of this substrate may not proceed via a reaction pathway involving a carbon deprotonation of an amine radical intermediate. Isootope effect measurements also established that the rate of inactivation of monoamine oxidase B by MPTP is unaffected by replacement of the C-6 methylene protons with deuterons, but is retarded by replacement of the C-2 methylene protons (\( K_m \) 1.9). The mechanism-based inactivation of monoamine oxidase B by MPTP, therefore, is likely to be mediated by a species derived from the enzyme-generated 2,3-dihydrodipyridinium oxidation product.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (1; MPTP) is a cyclic tertiary allylamine which selectively destroys the dopaminergic neurons of the substantia nigra and produces a clinical syndrome in humans which closely resembles idiopathic Parkinson's disease (Davis et al., 1979; Langston et al., 1983). This neurotoxic outcome is dependent on the monoamine oxidase B-catalyzed oxidation of MPTP to the 2,5-dihydrodipyridinium species MPDP* (2) which undergoes further oxidation to the pyridinium species MPP* (3) (Heikkila et al., 1984; Langston et al., 1984; Chiba et al., 1985). MPTP is the first reported tertiary amine with good monoamine oxidase substrate properties. This compound also is a mechanism-based inactivator of monoamine oxidase B (Salach et al., 1984).

In this paper, we report the results of deuterium isotope effect measurements for the monoamine oxidase B-catalyzed oxidation of MPTP to MPDP* using the deuterated analogs MPTP-6-d1 (1-d1), MPTP-6,6-d2 (1-d2), MPTP-2,2,6,6-d4 (1-d4), and MPTP-NCD3-2,2,6,6-d4 (1-d2). We have compared the isotope effects of the enzyme-catalyzed reaction with the corresponding values for the electrochemical oxidation of MPTP to MPDP*, a reaction thought to proceed via the amine radical pathway which has been proposed for the monoamine oxidase catalytic pathway (Silverman et al., 1980). We also have studied the effects of deuterium substitution on the rate of inactivation of monoamine oxidase B by MPTP.

**EXPERIMENTAL PROCEDURES**

*Chemicals—All chemicals and solvents were reagent grade. The perchlorate salt of MPDP* (Chiba et al., 1985), the HCl salt of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-6-d6 (Gessner et al., 1986), and 1-methyl-4-phenyl-5,6-dihydro-2-pyridone (4) (Wu et al., 1988) were synthesized as previously described. MPTP-HCl was purchased from Research Biochemicals, Inc., Wayland, MA; LiAlD4, NaBD4, and CD3NH2·HCl (all 98 atom % D) from Aldrich Chemical Co.; and paraformaldehyde-d2 (98 atom % D) from ICN Chemical & Radioisotope Division, Irvine, CA.

**Instrumentation**—1H NMR spectroscopy was performed on a GE 500-MHz Fourier transform spectrometer linked to a Nicolet 1180 computer; chemical shifts are reported in parts per million (ppm) relative to Me4Si. Low resolution chemical ionization (CI) mass spectra were run on a modified AIE MS902S instrument at 8 kV using isobutane (0.7 torr) as reagent gas and liquid secondary ion detection. A 25-ml H-type electrolysis cell with a glass carbon working electrode, a Pt auxiliary electrode, and a saturated calomel electrode served as the reference. A BAS CV-27 voltamnograph was used as the power source. Both quantitative and qualitative HPLC analyses were performed on a Beckman DU-50 spectrophotometer. A 25-ml H-type electrolysis cell with porous glass frits was employed for the electrochemical oxidation studies. The working and auxiliary electrodes were made of 1-inch platinum wire. A saturated calomel electrode served as the reference. A BAS CV-27 voltamnograph was used as the power source. Both quantitative and qualitative HPLC analyses were performed on a Beckman 114M chromatograph employing an HP model 1040A diode array detector. Separations were performed on an Altex Ultrasil SCX cation exchange column (10 pm x 25 cm x 4/6 cm inside diameter) at a flow rate of 1.8 ml/min using the following mobile phase: 92% 0.1 M acetic acid containing 0.075 M triethylamine·HCl and adequate formic acid to adjust the pH to 2.38% acetomethrine. Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, University of California, Berkeley, CA.

**Enzyme Preparation and Assay**—Monoamine oxidase B was isolated from bovine liver mitochondria by the method of Salach (1979).
and traces of hemeprotein were removed by centrifugation through a sucrose gradient (Weyer and Salach, 1981). The activity of the enzyme was determined spectrophotometrically at 250 nm using initial rate measurements (30-120 s) of the oxidation of 3.2 mM benzylamine. A unit of activity is defined as the amount of enzyme required to convert 1 μmol of benzylamine to benzaldehyde in 1 min. All enzyme incubations were performed at 30°C in 50 mM sodium phosphate buffer (pH 7.2) containing 0.2% Triton X-100 (w/v).

A suspension of 36 mg (0.86 mmol) of LiAlD₄ in ether was heated to reflux, and 80 mg (0.42 mmol) of benzylamine. A unit of activity is defined as the amount of enzyme initial rate measurements (30-120 s) of the oxidation of 3.2 mM benzylamine to benzaldehyde required to convert 1 pmol of benzylamine to benzaldehyde in 1 min.

The solution was filtered and dried over Na₂SO₄. The reaction mixture was heated to reflux for 3 h after which the excess LiAlD₄ was decomposed with cooling by the addition of H₂O (37 μl), 15% aqueous NaOH (37 μl), and H₂O (110 μl). The solution was filtered and dried over Na₂SO₄.

Ethereal HCl was added, and the resulting white precipitate was filtered and dried under vacuum to give 57.5 mg (65%) of the HCl salt of 1-d₄. Recrystallization from EtOH/Et₂O gave the analytical sample: m.p. 252°C; 1H NMR (D₂O) δ 7.58, 7.45-7.40 (m, 5H, ArH), 6.15 [s, 1H, C(5)H₃], 3.72 [bs, 1H, C(2)H₄], 3.39 [bs, 1H, C(2)H₄], 3.03 s (s, 3H, NCH₃), 2.95-2.85 (m, 2H, C(3)H₂); CIMS 176 (MH⁺, 100%).

C₃H₇D₄NCl
Calculated: C 51.95 H 7.05 N 9.68 Found: C 51.99 H 7.04 N 9.66

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-2,2,2,6,6-d₅ (1-d₅)—The d₅-substituted compound was synthesized following the literature procedure for N-methyl-2-aminohexane and (R)-3-hexanol (1956). A mixture of 3.5 g (10.9 mmol) paraformaldehyde-d₄, 4.0 g (5.9 mmol) of methylvamine HCl, and 5.2 ml of water was heated to reflux with stirring for approximately 30 min at which time a clear, homogenoue solution resulted. After cooling to 50°C, 7.6 ml (6.0 mmol) of α-methylstereine was added. With vigorous stirring, the two-phase mixture was heated to 95-100°C for 2.5 h which yielded a yellow, homogeneous solution. The reaction mixture was cooled to room temperature, 2.7 ml (6.1 mmol) of concentrated HSO₄ was added dropwise, and the resulting mixture was heated to 90-95°C for 3 h. The yellow water 5-dl. The solution was treated with water (50 ml) and extracted with benzene (2 x 30 ml). The aqueous layer was made basic (pH > 10) with aqueous 50% KOH and then was extracted with benzene (2 x 20 ml). The combined organic layers were dried over MgSO₄, and filtered, and the solvent was removed to leave 8.1 g of a deep yellow oil. The oil was dissolved in Et₂O, and ethereal HCl was added to give 5.25 g (45%) of the HCl salt of 1-d₄. Recrystallization from EtOH/Et₂O gave very pale yellow crystals: m.p. 247-248°C; 1H NMR (D₂O) δ 7.54 - 7.39 (m, 5H, ArH), 6.12 [s, 1H, C(5)H₃], 2.99 s (s, 3H, NCH₃), 2.91 [d, 1H, J = 15.4 Hz, C(3)H₃], 2.83 [d, 1H, J = 18.4 Hz, C(3)H₃]; CIMS 178 (MH⁺, 100%).

C₃H₇D₄NCl
Calculated: C 51.95 H 7.05 N 9.68 Found: C 51.99 H 7.04 N 9.66

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d₅ (1-d₅)—The d₅-substituted compound was synthesized by the same procedure as that used for the d₄ compound except that methyl-d₅-amine HCl replaced methylvamine HCl. The solid material obtained was recrystallized from EtOH/Et₂O to give 1.68 g (20%) of pale yellow crystals: m.p. 248-250°C; 1H NMR (D₂O) δ 7.57-7.45 (m, 5H, ArH), 6.11 [s, 1H, C(5)H₃], 2.90 [d, 1H, J = 18.3 Hz, C(3)H₃], 2.81 [d, 1H, J = 18.3 Hz, C(3)H₃].

C₃H₇D₄NCl
Calculated: C 51.95 H 7.05 N 9.68 Found: C 51.99 H 7.04 N 9.66

RESULTS AND DISCUSSION

The oxidation of MPTP to the 2,3-dihydropyridinium metabolite 2 could result from direct attack at C-6 or from initial attack at C-2 followed by rearrangement of the isomeric 2,5-dihydropyridinium intermediate 6 to 2 via the free base 7 (Scheme 1). Confirmation that the formation of MPDP⁺ occurs exclusively from oxidation of MPTP at C-6 was obtained from LSI mass spectral analysis of the α-cyano adduct 5 derived from a 60-min monoamine oxidase B incubation mixture of MPTP-d₅ which had been treated with NaCN. The mass spectrum of the isolate displayed an intense ion at m/z 281 which corresponds to the (MH⁻)-HCN-thioglycolyl⁺ species derived from 5-d₅. Weaker ions also were present at m/z 282 (MH⁻, 30%) and 172 [MH⁻ - HCN⁻, 60%]. The intensity of the m/z 282 ion corresponded to that expected for the ¹⁴C satellite peak of the 281 ion. Since no significant amounts of 5-d₅ were found, a pathway proceeding via 3 can be ruled out. Consequently, the monoamine oxidase B catalyzes the ring α-carbon oxidation of MPTP exclusively at the allylic C-6-position, isotope effect measurements will not be complicated by metabolic switching to the C-2 position.

Gessner et al. (1986) have studied the isotope effects for the monoamine oxidase B-catalyzed oxidation of MPTP-d₅, and reported values for δ¹⁴C (V) of 1.49 and δ¹⁴C(V)/K of 1.52. The significance of these values, however, is questionable since the C-6-position of MPTP is prochiral, and proton/deuteron
loss from MPTP-6-d1 could be stereoselective. This issue was addressed by CI mass spectral deuterium content analyses of the dihydropyridinium species 2 and 2-d1 (as the corresponding α-cyano adducts 5 (MH+ 199) and 5-d1 (MH+ 200)) derived from incubation mixtures of 100 μM MPTP-d1. Estimates of peak heights established that the d1/d0 product ratio decreased from approximately 1.5 at 10 min (20% conversion) to 1.1 at 60 min (60% conversion). These data suggest that the enantiomeric composition of the substrate changes with time due to the stereoselective loss of one of the enantiotopic hydrogen/deuterium atoms. This result is not surprising since the monoamine oxidase-catalyzed oxidation pathways of tyramine (Belleau et al., 1960) dopamine (Yu et al., 1986), and benzylamine (Yu and Davis, 1989) are all stereoselective.

The stereoselectivity observed for the monoamine oxidase B-catalyzed oxidation of MPTP precluded the possibility of measuring an intramolecular isotope for this reaction. As an alternative approach, we elected to examine the initial enzyme-catalyzed oxidation rates of the achiral 1, 1-d2, 1-d4, and 1-d9 substrates by spectroscopic measurement of MPDP+ formation over a substrate concentration range of 0.2–3.2 mM. The Lineweaver-Burk plots of the data obtained with 1 and 1-d9 (Fig. 1) are typical. The apparent isotope effects for the d1 analog were 3.55 on Vmax and 8.01 on Vmax/Km. The results summarized in Table I show that the Km, Vmax, V/K, and V(V/K) values are similar for all three deuterated substrates. Peaks observed in the HPLC tracings obtained from 30-min incubation mixtures of MPTP-d1 and MPTP-d9 showed MPDP+ and MPB as the only reaction products. Since these isotope effect values were generated from noncompetitive, intramolecular kinetic measurements, they may not reflect the intrinsic isotope effects for these substrates which, however, must be equal to or greater than the V(V/K) values (Northrup, 1975).

Since V(V/K) is large relative to V/V for this oxidation, the ratio of the rate of catalysis to the on/off rate of the enzyme-substrate complex is likely to be small or, as described by Northrup, the reaction has a "low commitment to catalysis" (Northrup, 1975). The suppression of V/V compared to V(V/K) suggests that the carbon-hydrogen bond cleavage step is only partially rate-determining. This result is not unexpected since one complete monoamine oxidase B catalytic cycle is composed of two half-reactions. In the first half-reaction, the substrate is oxidized, and the flavin moiety is reduced. In the second half-reaction, the product is released, and the reduced flavin is reoxidized. Rate constants for steps in both half-reactions appear in the expressions for both Vmax and Km while the rate constants for steps in the second half-reaction cancel out in the V(V/K) expression. The rates of the steps following the catalytic step, which appear in the expression for V', therefore, must be sufficiently slow to affect the rate of the overall reaction. This is consistent with stopped-flow studies which showed that the rate of the second (reoxidative) half-reaction is only 1.6 times that of the first half-reaction (Ramsay et al., 1987). The exact source of the differences in the Km values for the -d9 versus the deuterated substrates is not clear but must be due to differences in one or more of the variables that appear in the expression for Km (Northrup, 1975).

A proposed mechanism for the monoamine oxidase-catalyzed oxidation of amines (Scheme 2) involves initial transfer of one electron from the nitrogen lone pair to the flavin moiety of the enzyme to give the aminium radical 9 (Silverman et al., 1980). This potential rate-determining step is followed by α-carbon deprotonation and then transfer of a second electron from the resulting carbon-centered radical 10 to form the iminium product 11. This proposal is supported by studies on the mechanism-based inactivation of monoamine oxidase B by strained ring systems such as N-benzyl-N-cyclopropylamine (Vazquez and Silverman, 1985; Silverman and Zieske, 1985, 1986). Enzyme inactivation has been shown to result from active site alkylation by the radical species 13 which is formed by ring opening of the strained aminium radical 12. An isotope effect of 1.5 has been reported for the inactivation of monoamine oxidase B by N-benzyl-N-cyclopropylamine but only at low substrate concentrations (Silverman et al., 1980). The authors have interpreted these results as evidence that proton loss is partially rate-determining.
Mechanism of Monoamine Oxidase B-catalyzed Oxidation of MPTP

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Since electrochemical oxidations of amines proceed by initial one-electron abstraction from the nitrogen lone pair (Lindsay-Smith and Masheder, 1976; Lindsay-Smith and Mead, 1973; Masui et al., 1968), we examined the electrochemical oxidation of MPTP as a chemical model for the aminium radical pathway. The free base of MPTP (1 mM) was used since aqueous solutions of the hydrochloride salt were stable at applied potentials of up to 1.5 V. The cyclic voltammogram of a 1 mM MPTP solution at pH 10 showed an irreversible oxidation wave which peaked at 1.25 V. HPLC analyses of a solution which had undergone controlled potential electrolysis at 1.3 V for 60 min showed the presence of both MPDP\(^+\) (25 \(\mu\)M) and MPP\(^+\) (2 \(\mu\)M). No other oxidation products were observed in the chromatograms. In contrast, the electrochemical oxidation of N-methylpiperidine is reported to result in both ring carbon and N-methyl oxidation in a 60:40 ratio, respectively (Chiba and Takata, 1977). Apparently, the double bond in the MPTP ring greatly enhances the rate of hydrogen loss from the allylic position.

The isotope effect studies for the electrochemical oxidation of 1 mM MPTP employed the more readily available MPTP-d\(_4\) analog. The rates were calculated from the slopes of the plots of "total" MPDP\(^+\) formed, i.e. the sum of MPDP\(^+\) and MPP\(^+\), against time. The \(K\) value for this reaction was 1.35. Enzyme-catalyzed reactions which are thought to proceed by a similar mechanism include the cytochrome P-450-catalyzed N-demethylation of N,N-dimethylaniline which displays an intramolecular \(K\) value of 3.05 (Miwa et al., 1983) and the N-demethylation of N,N-dimethylphenyltermine which displays an intramolecular \(K(V/K)\) value of 1.6 to 2.0 (Miwa et al., 1980). On the other hand, the large intramolecular \(K\) isotope effects observed for the horseradish peroxidase-catalyzed oxidative N-demethylation of N,N-dimethylaniline (8.8-10.1) (Miwa et al., 1983) and for the cytochrome P-450-catalyzed benzyllic oxidation of 1,3-diphenyipropane (11) (Hjelmeland et al., 1977) have been interpreted as evidence to support a reaction pathway involving hydrogen atom abstraction. The oxidative deamination of benzylamine by peroxidase (\(K = 7\)) also is thought to proceed by a hydrogen atom abstraction mechanism (Wei and Stewart, 1966).

The large \(K(V/K)\) values observed for the monoamine oxidase B-catalyzed oxidation of the various MPTP deuterated analogs indicate that C-H bond cleavage is rate-determining and also that the transition state in this transformation is of high symmetry. Furthermore, a comparison of the isotope effect values attributed to an aminium radical deprotonation pathway versus those linked to a hydrogen atom abstraction pathway suggests that the mechanism for the monoamine oxidase B-catalyzed oxidation of MPTP is more likely to proceed by hydrogen atom abstraction than deprotonation of an aminium radical. A similar conclusion may be reached with the monoamine oxidase B-catalyzed oxidation of benzylamine which is reported to have isotope effects of 6.7 on \(V_{max}\) and 6.3 on \(V_{max}/K_m\) (Husain et al., 1982). On the other hand, the stereospecific monoamine oxidase B-catalyzed oxidation of (R)-dopa-mine\(\alpha\)-d\(_1\) showed isotope effects of 3.1 and 2.8 on \(V_{max}\) and on \(V_{max}/K_m\), respectively (Yu et al., 1986). The corresponding values for the deamination of 2-phenethylamine-1,1-d\(_2\) are 1.44 on \(V_{max}\) and 1.10 on \(V_{max}/K_m\) (Husain et al., 1982). These lower isotope effect values may be consistent with the aminium radical pathway proposed by Silverman and Zieske (1986) in which case we would suggest that the monoamine oxidase B-catalyzed oxidation of benzylc and allylic positions may proceed by an alternative pathway.

Attempts to gain further insights into the monoamine oxidase B-catalyzed oxidation of MPTP were pursued through kinetic analysis of the inactivation pathway. If MPTP were to be converted to the corresponding radical cation \(14\), a
subsequent step could involve rearrangement to the stabilized carbon-centered radical 15, a species analogous to the radical 13 which Silverman proposed was responsible for the inactivation of monoamine oxidase B by N-benzyl-N-cyclopropylamine (Scheme 2). If one assumes that the enzyme-inactivating species is formed from the same intermediate that leads to product formation, then substitution of hydrogen with deuterium at the 6-position of MPTP would be expected to lead to an increase in the rate of inactivation of monoamine oxidase B. That is, an inverse isotope effect on the rate of inactivation would be observed as a result of the normal isotope effect on the process leading to MPDP+. Fig. 2 displays the plots of remaining monoamine oxidase B activity versus time following incubations with 2 mM MPTP, MPTP-d₈, and MPTP-d₆. Pseudo-first order kinetics were observed with all three compounds. The rates of inactivation were the same with MPTP-d₆ and MPTP-d₈, whereas the corresponding rate of inactivation with MPTP-d₄ was 1.9 times slower than that observed with the -d₈ and -d₆ substrates. This result suggests that enzyme inactivation must occur after C(2)-H bond cleavage, i.e., after MPDP+ formation. Consistent with this proposal is the report that MPDP+ also is a substrate for and a mechanism-based inactivator of monoamine oxidase B (Singer et al., 1986).

A possible pathway to account for the inactivation of monoamine oxidase B by MPTP involves initial hydrogen atom abstraction from the free base 7 of MPDP+ to yield the stabilized radical intermediate 19 that could alkylate the enzyme to produce 20. This adduct would be expected to hydrolyze to 21 and methylamine (Scheme 3). In support of this possibility are recent results from our laboratory which have documented the monoamine oxidase B-dependent formation of methylamine from MPTP (Castagnoli et al., 1988). We know, however, that methylamine also is produced from MPDP+ following a sequence of reactions that consumes 3 mol of MPDP+ and produces the hexahydroisoquinoline 22 (Leung et al., 1989). Studies currently in progress are designed to clarify further the mechanistic features of the interactions of MPTP with monoamine oxidase B and to characterize the reaction sequence leading to the inactivation of the enzyme.

REFERENCES


