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Interactions of the neurotoxic amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine with monoamine oxidases

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a thermal breakdown product of a meperidine-like narcotic used by drug abusers as a heroin substitute, produces Parkinsonian symptoms in humans and primates. The nigrostriatal toxicity is not due to MPTP itself but to one or more oxidation products resulting from the action of monoamine oxidase (MAO) on this tertiary allylamine. Both MAO A and B catalyse the oxidation of MPTP to the 1-methyl-4-phenyl-2,3-dihydropyridinium species ($MPDP^+$), which undergoes further oxidation to the fully aromatic 1-methyl-4-phenylpyridinium species (MPP^+). These bio-oxidations are blocked by selective inhibitors of MAO A and B. Additionally, MPTP, $MPDP^+$ and MPP^+ are competitive inhibitors of MAO A and B. The A form of the enzyme is particularly sensitive to this type of reversible inhibition. Both MAO A and B also are irreversibly inactivated by MPTP and $MPDP^+$, but not by MPP^+. This inactivation obeys the characteristics of a mechanism-based or 'suicide' process. The inactivation, which is accompanied by the incorporation of radioactivity from methyl-labelled MPTP, is likely to result from covalent modification of the enzyme.

INTRODUCTION

In 1983, Langston *et al.* reported that the Parkinsonian symptoms observed in a group of street drug users was caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Fig. 1). Subsequent studies have documented that MPTP is an effective neurotoxin that selectively destroys nigrostriatal cells in susceptible species (Burns *et al.*, 1983; Langston *et al.*, 1983; Kolata, 1983). The close similarities between MPTP-induced Parkinsonism and idiopathic Parkinson's disease have led to a concerted effort to elucidate the molecular events involved in the mechanism of neurotoxicity of MPTP (Markey *et al.*, 1986).

Although MPTP is lipophilic and crosses the blood/brain barrier, its inherent chemical stability suggested to us that its neurotoxic properties might be mediated by chemically reactive metabolites generated within the central nervous system. The observation that rat brain mitochondrial preparations catalysed the oxidation of MPTP to the 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and 1-methyl-4-phenylpyridinium (MPP^+) species (Fig. 1) *in vitro* provided support for this proposal (Chiba *et al.*, 1984; Castagnoli *et al.*, 1985). Since this catalytic activity of brain mitochondrial preparations was inhibited by deprenyl but not clorgyline, we proposed that the oxidation of MPTP was catalysed by MAO B (Chiba *et al.*, 1984). These findings prompted studies *in vivo*, which have shown that pretreatment with inhibitors of MAO B completely protects susceptible animals against the neurotoxic effects of MPTP (Heikkila *et al.*, 1984; Langston *et al.*, 1984).

The evidence summarized above supports the suggestion that brain MAO catalyses the bioactivation *in vivo* of MPTP to neurotoxic metabolites. In order to characterize this process more fully, we have examined the interaction of this cyclic tertiary allylamine with highly purified preparations of both MAO A and MAO B. Parallel experiments with $MPDP^+$, the two-electron oxidation product of MPTP, also have been conducted, in part because of the chemical reactivity of this electrophilic dihydropyridinium species (Chiba *et al.*, 1985*a*; Peterson *et al.*, 1985). The present paper is a detailed account of studies that have been reported briefly in two preliminary communications (Salach *et al.*, 1984; Singer *et al.*, 1985).

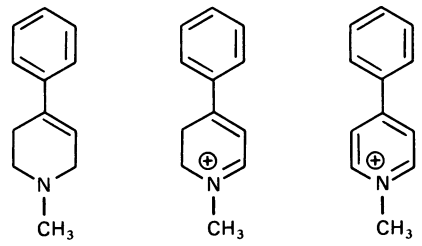

Fig. 1. MPTP and its oxidation products, $MPDP^+$ and MPP^+

EXPERIMENTAL

Materials

MAO B from ox liver mitochondria was isolated by the procedure of Salach (1979), and traces of haem protein

Abbreviations used: MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium species; MPP^+, 1-methyl-4-phenylpyridinium species.

were removed by centrifugation in a sucrose gradient (Weyler & Salach, 1981). MAO A was purified to > 90% homogeneity from human placental mitochondria (Weyler & Salach, 1985). [*methyl*-^{3}H]MPTP (85 Ci/mmol) was purchased from New England Nuclear, MPTP from the Aldrich Chemical Co., and 1-methylpyridinium iodide and deprenyl were gifts of Dr. Sanford Markey, National Institutes of Health, Bethesda, MD, U.S.A., and Dr. Joseph Knoll, Semmelweiss University, Budapest, Hungary, respectively. The sources of other chemicals were as reported previously (Salach *et al.*, 1984; Singer *et al.*, 1985).

Methods

The activities of MAO A and B were determined spectrophotometrically at 30 °C by using strict initial-rate measurements (30–120 s reaction periods), the former with kynuramine and the latter with benzylamine as substrate (Salach, 1979; Weyler & Salach, 1985). Absorption spectra were recorded on a Cary 219 or a Hewlett–Packard diode array instrument. Oxygen uptake was monitored with a Clark oxygen electrode at 30 °C. For rapid separation of protein-bound and free solutes, a short centrifugation through a Sephadex G-25 column was used (Penefsky, 1977).

RESULTS AND DISCUSSION

Enzymic oxidation of MPTP and MPDP$^+$

Both MAO A and MAO B catalyse the oxidation of MPTP to MPDP$^+$ at substantial rates. Although predicted by our results with brain mitochondrial preparations (Chiba *et al.*, 1984), the observed oxidation rates were impressive given that tertiary amines are considered to be only very slowly oxidized in the presence of MAO (Blaschko, 1963). MAO B catalysed the oxidation of MPTP at a rate of 1.71 nmol/min per μg of enzyme, 40% of that observed with benzylamine, the most rapidly oxidized substrate for the enzyme in steady-state assays. The K_m value for this reaction (0.30 mM) was even lower than that for benzylamine (0.38 mM). The rate of oxidation by MAO A also was substantial (0.11 nmol/min per mg of enzyme), and the K_m value, 0.14 mM, is similar to that for kynuramine, a preferred substrate (Salach *et al.*, 1984).

Our initial studies on the rates of the MAO-catalysed oxidation of MPTP suggested a stoichiometric conversion into MPDP$^+$. This product is a relatively unstable molecule which, at millimolar concentrations, undergoes spontaneous disproportionation to MPTP and MPP$^+$ (Peterson *et al.*, 1985). An examination of the concentration-dependence of this bimolecular reaction, monitored spectrally by observing the disappearance of MPDP$^+$ at 343 nm and the appearance of MPP$^+$ at 290 nm, revealed that disproportionation occurred at an insignificant rate at an MPDP$^+$ concentration of 50 μM. Instead, the molecule underwent slow autoxidation ($t_{\frac{1}{2}}$ = 12 h at 30 °C) to give a stoichiometric yield of MPP$^+$. The rate of this reaction was not affected by 0.05 μM concentrations of Fe^{2+}, Fe^{3+} or Cu^{2+} ions, in the presence or absence of 1 mM-EDTA. However, both MAO A and MAO B accelerated the oxidation of MPDP$^+$. In a typical experiment with 50 μM-MPDP$^+$ and MAO B, the rate of MPDP$^+$ oxidation was 0.93 mol/min per mol of enzyme, and this was decreased to 0.23 mol/min per mol of enzyme by the addition of deprenyl. MAO A catalysed the oxidation of MPDP$^+$ at a comparable rate, and this reaction was inhibited 50–75% by clorgyline. The oxidation rates for MPDP$^+$ were relatively low, partly because the substrate concentration used to avoid spontaneous disproportionation (50 μM) is most likely well below the K_m value, assuming that this is comparable with that for MPTP (Salach *et al.*, 1984). We therefore monitored the oxidation polarographically, at a higher MPDP$^+$ concentration (480 μM) that was still below the range where chemical disproportionation predominates. The data (Table 1) show significant catalytic activity for MAO B, although the rate of oxidation of MPDP$^+$ was far below that of MPTP. Note that these experiments were conducted at a single concentration of substrate and O_2 and thus are not $V_{max.}$ values. However, we tentatively conclude that the rate of oxidation of MPDP$^+$ in the presence of MAO B is about 1% of the corresponding rate of oxidation of benzylamine, a preferred substrate for the enzyme. The currently held view that the toxicity of MPTP may be mediated specifically by MPP$^+$ (Javitch *et al.*, 1985; Chiba *et al.*, 1985*b*) lends importance to this catalytic activity, even though it is low compared with that observed with MPTP itself.

Table 1. Enzymatic oxidation of 2,3-MPDP$^+$

MAO B (0.63 nmol), dissolved in 50 mM-phosphate, pH 7.2, containing 0.2% (w/v) Brij 35, was diluted in the chamber of the oxygen electrode to 2 ml final volume. Deprenyl treatment was for 30 min at 30 °C with 0.3 mM-inhibitor. Benzylamine and MPDP$^+$ were present at concentrations of 3.33 mM and 480 μM respectively. The low concentration of MPDP$^+$ was chosen to minimize disproportionation, but, as a result, the rate measured was not $V_{max.}$. The temperature was 30 °C.

Substrate	Inhibitor	O_2 uptake (μatoms/min per mg)	Catalytic-centre activity (mol of MPDP$^+$/min per mol of enzyme)
Benzylamine	None	4320	630
	Deprenyl	0	0
MPDP$^+$	None	40.8	6
	Deprenyl	6.0	0.9

Reversible inhibition of MAO by MPTP and its oxidation products

MPTP and its two oxidation products, MPDP$^+$ and MPP$^+$, are effective competitive inhibitors of MAO, particularly of MAO A (Singer *et al.*, 1985). With kynuramine as the assay substrate, the MAO A K_i values for MPTP, MPDP$^+$ and MPP$^+$ were 18, 6 and 3 μM respectively. The reverse order of inhibitory potency was observed for MAO B, which is competitively inhibited 1.5–2 orders of magnitude less effectively with benzylamine as substrate. Fuller & Hemrick-Luecke (1985) also observed competitive inhibition of MAO B activity in rat brain preparations by MPTP, but reported that MAO A is only irreversibly inhibited by the amine. This apparent discrepancy may be due to methodological differences, since, as we have pointed out, MAO assays based on the use of radiolabelled substrates are unsuited for initial-rate measurements and could preclude detection of a

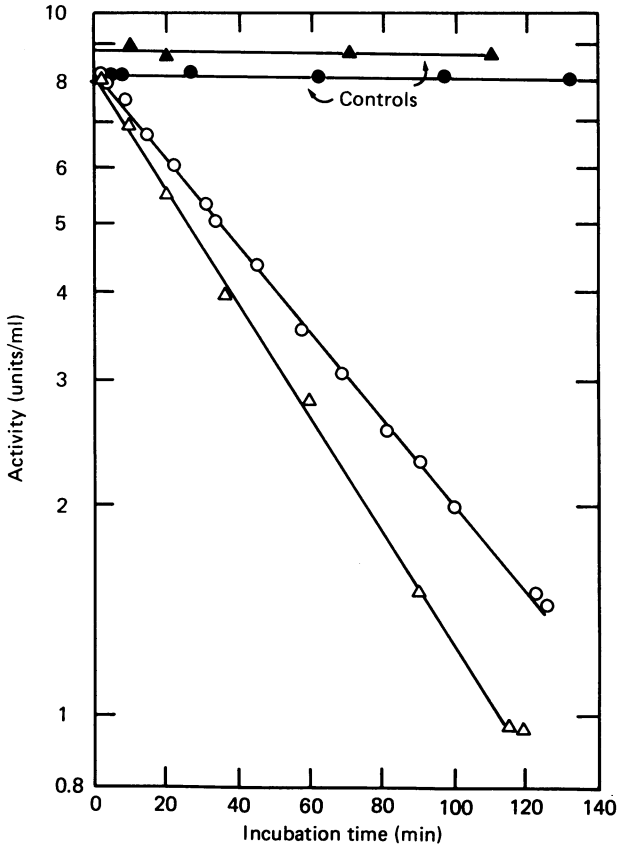

Fig. 2. Kinetics of the inactivation of MAO A and B by MPTP

MAO A was incubated at 30 °C with 5 mM-MPTP in the standard assay mixture but without kynuramine (△). Control samples were similarly incubated without MPTP (▲). Portions (10 μl each) were periodically removed and added to 0.99 ml of the standard assay mixture containing 1 mM-kynuramine for estimations of activity. MAO B was similarly incubated in the absence of benzylamine with (○) or without (●) 2 mM-MPTP. Portions of volume 10 μl were taken for assay of enzyme activity with 3.3 mM-benzylamine.

competitive phase of inhibition before irreversible inactivation of the enzyme (Singer, 1985).

Mechanism-based irreversible inactivation

Time-dependent effects were observed when MAO A or MAO B was incubated with either MPTP or $MPDP^+$. Portions were removed at frequent intervals, diluted with a large excess of substrate (kynuramine or benzylamine respectively) to reverse competitive inhibition and enzyme activity assayed. During the initial phase, full activity was recovered; presumably the high ratio of substrate to inhibitor concentration overcomes the competitive effects of MPTP or $MPDP^+$. In the second phase, enzyme activity progressively declined. This loss of catalytic activity was not reversed by passage through a Sephadex column equilibrated with the normal substrates. Fig. 2 shows this time-dependent irreversible inactivation of MAO A and MAO B by MPTP. The first-order rate constants for inactivation by MPTP (5 mM) were 1.0×10^{-2} and 3.4×10^{-2} min^{-1} for MAO A and MAO B respectively. The linearity of the exponential plots together with the protection against inactivation observed when normal substrates were added before the addition of MPTP, was indicative of a mechanism-based or 'suicide' inactivation of MAO (Singer, 1985). Similar conclusions have been reached by Fuller & Hemrick-Luecke (1985) in studies of the effects of MPTP on rodent brain MAO B. $MPDP^+$ also caused a time-dependent loss of activity with first-order rate constants for inactivation of 0.85×10^{-2} (5.1 mM) and 2.2×10^{-2} (2.9 mM) min^{-1} for MAO A and MAO B respectively. No time-dependent inactivation was observed with MPP^+ as anticipated, since this quaternary compound should not be oxidized further by these enzymes, a prerequisite for mechanism-based inhibition.

Characteristics of the time-dependent inactivation

Time-dependent, irreversible enzyme inhibition may involve the covalent binding of metabolically generated reactive intermediates to the enzyme. The incubation of [^{3}H]MPTP with MAO B resulted in extensive association of radiolabel with the protein, as judged by gel-exclusion chromatography on Sephadex G-25. In order to separate radiolabelled material associated with the enzyme by non-covalent binding forces, the protein was precipitated with 10 vol. of cold acetone. The resulting protein precipitate contained little or no radioactivity in samples taken immediately after mixing [^{3}H]MPTP with MAO B, but the amount increased progressively during time-dependent inactivation to reach a maximum of 5 mol of radioactive product bound/mol of enzyme. This ratio may reflect the high partition coefficient associated with the inactivation process. Since only a small fraction of the electrophilic intermediates generated reacts at the catalytic centre, this species may dissociate from the active site and react with more distant nucleophilic functionalities of the enzyme. Similar studies have not yet been conducted with MAO A owing to the scarcity of purified enzyme.

Further characterization of the inactivation process was afforded by timed spectral analyses of the reaction between MPTP and MAO B (Fig. 3). The spectrum of the fully active, oxidized enzyme ($\lambda_{max.}$ 460 nm) is shown in the inset. The first changes evident (30 s) were a loss of the 460 nm chromophore due to reduction of the 8α-*S*-cysteinyl FAD moiety and the appearance of a band at 343 nm representing the formation of the 2-electron oxidation product, $MPDP^+$. The intensity of the 343 nm band increased with time, but after 15 min began to decline, presumably as a result of the further oxidation of $MPDP^+$ to MPP^+. The spectral analyses revealed a shoulder at 410 nm after 15 min, which progressively increased in intensity. Since this absorption band may extend beyond 460 nm, it was not possible to determine if the flavin moiety undergoes reoxidation at a later time.

Fig. 4 shows a comparison of the changes in A_{410} with loss of enzyme activity as a function of time. Although suggestive of a relationship between the two events, the data do not necessarily indicate that enzyme inactivation is caused by (or reflected in) the formation of the compound absorbing at 410 nm. The molecular nature of this species remains to be determined. However, it binds to the enzyme tightly enough to remain with the protein during gel-exclusion chromatography and precipitation with trichloroacetic acid.

Indirect evidence that a covalently bound flavin group is not involved in the inactivation of MAO B and MPTP is that, upon purification, the flavin peptides obtained by

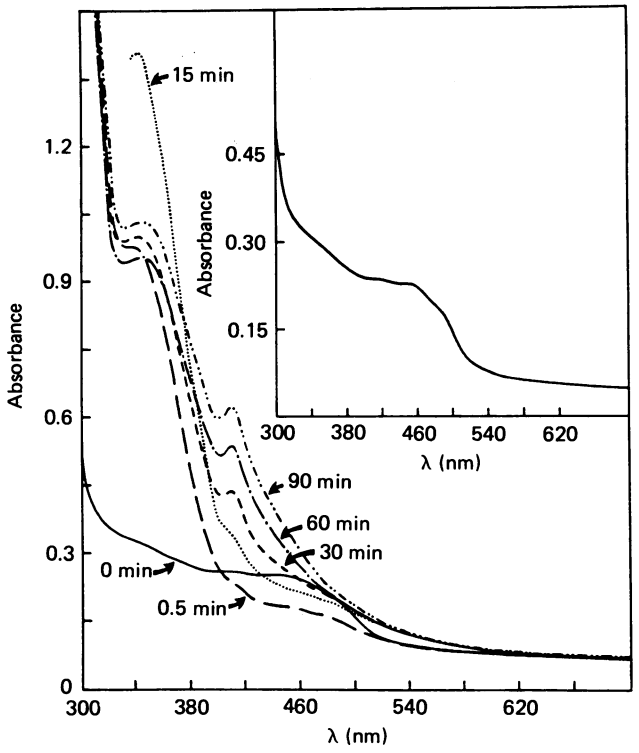

Fig. 3. Absorption changes occurring during the inactivation of MAO B by MPTP

The enzyme (3.8 ml, 2.3 mg/ml) in 50 mM-Hepes buffer, pH 7.2, containing 2 mg of Triton X-100/ml, was incubated with 4.9 mM-MPTP at 30 °C. At the times shown, 0.5 ml aliquots were rapidly chilled and centrifuged through 5 ml columns of Sephadex G-25 equilibrated with the same buffer. An equal volume (0.5 ml) of the same buffer was used to elute the protein from the columns. Spectra were taken immediately. Loss of activity and protein concentration were measured as described above. The protein concentrations did not vary by more than 5%.

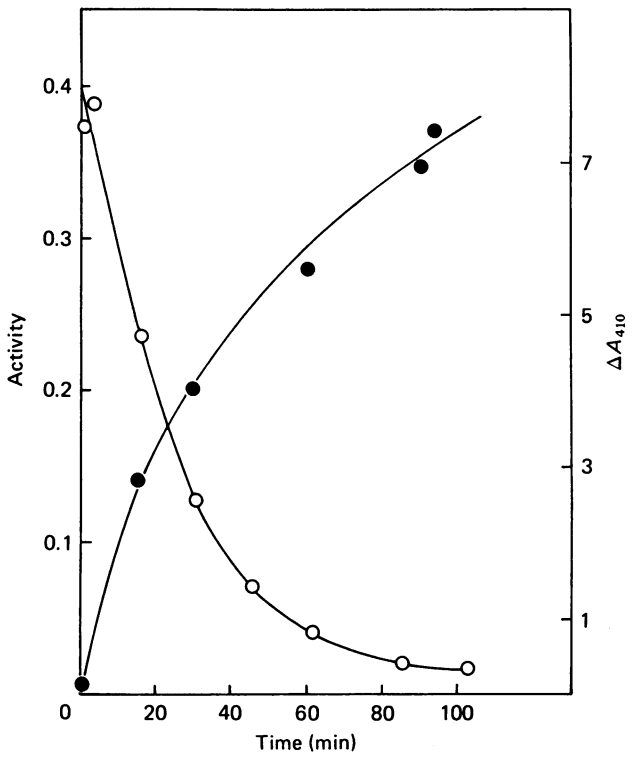

Fig. 4. Time-dependency of MAO B inactivation by MPTP (○) and the increase in absorbance at 410 nm (●)

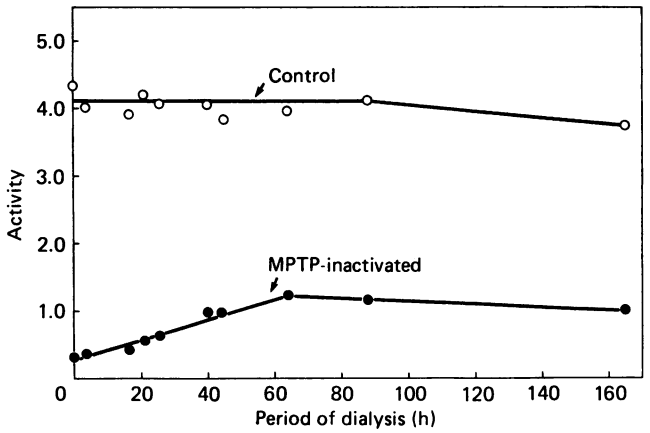

Fig. 5. Partial reactivation of MPTP-inactivated MAO B by dialysis

A sample of the enzyme was inactivated with 5 mM-MPTP at 30 °C. It then was centrifuged rapidly through a Sephadex G-25 column to remove uncombined MPTP and products, as in the experiments of Fig. 3. Aliquots (1 ml each) were then dialysed against 100 vol. of 50 mM-phosphate buffer, pH 7.2, containing 2 mg of Triton X-100/ml at 0 °C, with frequent changes of the dialysing fluid. Samples were removed and assayed with 3.3 mM-benzylamine at the times shown. The control was similarly treated, except for preincubation with MPTP.

proteolysis of the inactivated enzyme display spectral properties typical of cysteinylflavin peptides. It is known that adducts formed via covalent interactions with N-5 or C-4a of the flavin show markedly altered absorption spectra (Ghisla *et al.*, 1973). The only other nucleophilic group known to be present at the active site of MAO is the -SH group. Thiol groups of proteins characteristically form adducts with carbonyls (thiohemiacetals or thiohemiketals), which are usually stabilized by the tertiary structure of the native protein, but which undergo gradual dissociation once the protein is denatured. For example, mechanism-based inactivation of MAO B by *trans*-phenylcyclopropylamine (Paech *et al.*, 1980) involves the formation of a thiohemiacetal adduct between the active-site thiol and cinnamaldehyde (Silverman, 1983). Such adducts tend to dissociate slowly, even with prolonged dialysis of the native enzyme against preferred MAO substrates (Hellerman & Erwin, 1968). In this context it is noteworthy that the activity of MPTP-inactivated MAO B could be partially restored by dialysis over a period of 70 h, during which time control samples of the enzyme retained full activity (Fig. 5).

Despite over half a century of research on MAO, a re-examination of the substrate specificities of both forms of the enzyme appears necessary, particularly with regard to the oxidation of compounds related to MPTP. This matter assumes additional significance in view of suggestions that MPTP may be a model compound for chemicals in the environment which may be contributory factors in the development of idiopathic Parkinson's

disease (Calne & Langston, 1983; Langston *et al.*, 1983; Lewin, 1985; Snyder & D'Amato, 1985). It is clear that MAO A and B not only catalyse the oxidation of biogenic amines such as dopamine (3,4-dihydroxyphenethylamine), noradrenaline and serotonin, but also play an important role in the processing of xenobiotics. This may result in detoxification or, as is the case of MPTP, the conversion of a biologically unreactive molecule into highly toxic metabolites. The chemical reactivity of intermediates formed during the MAO-catalysed oxidations of MPTP presumably is responsible for the mechanism-based inactivation of the enzymes. Whether such oxidation products share a common molecular identity with those responsible for the selective cytotoxic effects on central dopaminergic neurons remains to be elucidated.

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