Mechanism-based inhibition of monoamine oxidase by 3-aryl- Δ^3 -pyrrolines

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The compound 1-methyl-3-phenyl- Δ^3 -pyrroline and its 4-chlorophenyl analogue appear to act as irreversible inhibitors of monoamine oxidase B (MAO B) in mitochondria of rat liver. The compounds are metabolized by MAO B and concomitantly inhibit the enzyme in what seems to be a two-step process. The

metabolic end product of this process is the corresponding pyrrole. This inhibition process is considered in the light of possible intermediates formed during the enzymic oxidation, and comparisons are made with the structurally analogous neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

INTRODUCTION

Monoamine oxidase (MAO) [amine: oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4.] acts on primary, secondary and tertiary aralkylamines. The last are generally very poor substrates, though notable exceptions are 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its analogues [1]. It seems likely that the presence of the β,γ double bond in the hetero ring of these compounds, an allylic structure, facilitates attack by the enzyme on the methylene group adjacent to the

unsaturated centre, though it may be noted that the allylic tertiary amine *N,N*-dimethylcinnamylamine (an open-chain analogue) is not a good substrate for MAO, though it does inactivate MAO B [2]. MPTP is a prototoxin, being converted via oxidation by MAO B to the cation 1-methyl-4-phenylpyridinium (MPP⁺). This interferes with the electron-transport chain by inhibiting Complex 1 in mitochondria (see [3] and references cited therein). This is likely to be one of the reasons for the destruction of nigrostriatal dopaminergic cells, leading to the severe Parkinsonian syndrome that follows ingestion of MPTP [4].

It was shown recently that Δ^3 -pyrroline inactivates plasma amine oxidase (PAO; EC 1.4.3.6) by reaction with the topaquinone cofactor [5]. Those authors suggested that pyrroline could be used to discriminate between PAO and the flavoenzyme MAO, since it should act as a substrate for the latter. This has been confirmed very recently in a study of the oxidation of substituted pyrrolines by MAO from bovine liver [6] and in the present work, using rat liver mitochondria as a source of MAO. However, we also show that although 3-aryl-substituted Δ^3 -pyrrolines are metabolized by MAO B, they inactivate the enzyme in what appears to be a two-stage process. The compounds do not appear to be metabolized by MAO A in rat liver, although they were found to inhibit 5-HT oxidation. The 3-phenyl compound bears close structural similarity to MPTP (see Scheme 1) in that the Δ^3 -pyrroline ring can be seen as a five-membered analogue of the heterocyclic ring in MPTP, though the chemistries of these two ring systems show substantial differences. It may also be noted that, like 4'-chloro-MPTP [1], the analogous 1-methyl-3-(4-chlorophenyl)- Δ^3 -pyrroline described here, is also metabolized by MAO B.

MATERIALS AND METHODS

Synthesis of pyrrolines

1-Methyl-3-(4-chlorophenyl)pyrroline was provided by Dr. P. T. Gallagher, Eli Lilly Research Centre, Windlesham, Surrey, U.K. 1-Methyl-3-phenylpyrroline was prepared as described by Gallagher et al. [7]. The pyrrolines were converted into their corresponding Δ^3 -pyrrolines by partial reduction with Zn/HCl using a general method described by Andrews and McElvain [8], but it was found necessary to include ethanol in the reaction mixture to dissolve the pyrrole derivatives used here. At the end of the reaction, the ethanol was removed by evaporation *in vacuo*. The aqueous acidic residue was washed with ethyl acetate

Scheme 1 Possible pathways for action of MAO on pyrrolines

¹Enz-Nu- represents a possible nucleophilic group on the enzyme.

Abbreviations used: MAO, monoamine oxidase; MPDP⁺, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP⁺, 1-methyl-4-phenylpyridinium; PAO, plasma amine oxidase; 5-HT, 5-hydroxytryptamine; TFA, trifluoroacetic acid.

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Figure 1 HPLC profiles of pyrrolines and pyrroles

(a) Purified sample of 1-methyl-3-phenyl- Δ^3 -pyrroline on a Bio-Rad C_{18} reverse-phase column. Conditions: solvent A, water/0.05% TFA; solvent B acetonitrile/0.05% TFA. Gradient 20–80% B over 30 min; flow rate 1 ml/min. (b) Purified sample of 1-methyl-3-(4-chlorophenyl)- Δ^3 -pyrroline on a Waters C_{18} semi-preparative column; flow rate, 1.5 ml/min; gradient, as in (a). (c) Continuous line: diethyl ether extract of reaction mixture from incubation of 1-methyl-3-(4-chlorophenyl)- Δ^3 -pyrroline (0.5 mM) with 300 μ g of mitochondrial protein for 35 min. The peak at approx. 10 min is the pyrroline. The overlaid dotted line represents an authentic sample of 1-methyl-3-(4-chlorophenyl)pyrrole extracted in the same manner. HPLC conditions were as for (b). (d) Diethyl ether extract of mitochondrial suspension as in (c), but in the absence of the pyrroline. HPLC conditions were as in (b) and (c). Diethyl ether extracts from an analogous experiment with 1-methyl-3-phenylpyrroline gave similar results, i.e. a peak co-incident with 1-methyl-3-phenylpyrrole.

to remove unchanged pyrrole, then adjusted to pH 10 with NaOH. The pyrroline was then extracted with ethyl acetate. The organic phase was washed twice with water, dried over anhydrous $MgSO_4$ and evaporated. The residue was converted into a hydrochloride. Reverse-phase HPLC on a Bio-Rad C_{18} column gave a single peak in each case (Figures 1a and 1b).

1H Nmr: 1-methyl-3-phenyl- Δ^3 -pyrroline. δ (p.p.m.) [(2H)dimethyl sulphoxide] 2.95 [3 H, s, N- CH_3], 4.15 [1 H, d, CH_2], 4.3 [1 H, d, CH_2], 4.4 [1 H, d, CH_2], 4.65 [1 H, d, CH_2], 6.45 [1 H, s, olefinic CH], 7.3–7.6 [5 H, m, C_6H_5]; 1-methyl-3-(4-chlorophenyl)- Δ^3 -pyrroline: δ (p.p.m.) [(2H)dimethyl sulphoxide] 2.9 [3 H, s, N- CH_3], 4.2 [2 H, s, CH_2], 4.45 [2 H, s, CH_2], 6.5 [1 H, s, olefinic CH], 7.4–7.6 [4 H, m, C_6H_4].

Enzymic studies

Mitochondria from rat liver were isolated as described previously [9]. The protein content was measured by the method of Markwell et al. [10], with BSA as standard.

All experiments were carried out in triplicate. MAO activity was measured using (*E*)-cinnamylamine as substrate [11], as follows. Mitochondrial protein (300 μ g) in 100 mM sodium/potassium phosphate buffer, pH 7.5 (0.95 ml) was incubated in a cuvette (pathlength 10 mm) in a Perkin–Elmer Lambda 5 spectrophotometer at 30 °C. The reaction was started by adding (*E*)-cinnamylamine (50 μ l of a 2 mM solution in water) and the progress of the reaction [formation of (*E*)-cinnamaldehyde] was monitored at 290 nm. Initial rates were calculated by using an ϵ_{290} value of 24 500 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$.

The action of MAO on the pyrrolines (I) and (II) (Scheme 1) (0.1 mM) was first examined by incubating them with mitochondrial protein (150 μ g) under conditions as described in the cinnamylamine assay, and scanning of their UV spectra (240–320 nm) at 5 min intervals. This showed a change in each of the spectra, characterized by an increase in absorbance in the 280–300 nm region and a decline in the peak absorbance of the pyrrolines at approx. 250 nm. These spectral changes were unaffected by preincubation of mitochondria for 15 min with the

Figure 2 Spectral changes in pyrrolines (I) and (II) (0.1 mM) produced by the action of mitochondrial MAO (150 μ g of protein)

The pyrrolines were incubated with protein in a 1 cm cuvette and spectra were collected at 5 min intervals for a total of 45 min. Conditions: 100 mM phosphate buffer, pH 7.5; temperature 30 °C; total assay volume 1 ml. Spectra from left to right are: pyrroline (I), pyrroline (I) after preincubation of mitochondria with MAO inhibitor (*R*)-deprenyl, pyrroline (II) and pyrroline (II) after preincubation of mitochondria with MAO inhibitor (*R*)-deprenyl. The increase in absorbance at about 280–300 nm in uninhibited samples is accompanied by a decrease in the peak absorbance of the pyrrolines (approx. 248 nm), suggesting that they are converted into a species with a more extended chromophore, consistent with formation of the corresponding pyrroles.

MAO A inhibitor clorgyline (100 nM), but were prevented by similar pretreatment with the MAO B inhibitor (*R*)-deprenyl (500 nM). We have shown in many independent experiments that these conditions result in selective inhibition of the preferred form of MAO in rat liver mitochondria, without causing inhibition of the other form.

In further experiments, the pyrrolines (final concn. 0.5 mM) were incubated with mitochondrial protein as described above and the progress curves at 280 nm (I) or 290 nm (II) were recorded. This experiment was repeated on samples of the enzyme preparation that had been preincubated with clorgyline or (*R*)-deprenyl as described above.

To assess the nature of the interaction of the pyrrolines with MAO, 300 μ g samples of mitochondrial protein were incubated with compound (I) (0.5 mM) or compound (II) (0.5 mM) for 1 h and for 3 h in Eppendorf-style tubes under the conditions described above (pH 7.5, 30 °C, total vol. 1 ml). The samples were then centrifuged at 17000 *g* for 2 min and the pellets were washed three times in phosphate buffer (3 $\times$ 1 ml) by resuspension and centrifugation. Pellets were suspended in buffer (0.95 ml) and assayed for MAO activity using (*E*)-cinnamylamine as described above. Controls, without the pyrroline derivative, were treated in the same way.

In a separate experiment, mitochondrial samples that had been incubated with (I) and then washed as described above, were preincubated with the MAO A inhibitor clorgyline (100 nM) as described above before assaying for MAO activity.

K_m and V_{max} values for (I) and (II) acting as substrates for MAO B were obtained by spectrophotometric measurements, in triplicate, of initial rates of increase of absorbance at either 280

or 290 nm respectively, using a range of concentrations, under conditions as described for assay with (*E*)-cinnamylamine. Initial rates were derived from tangents drawn to the progress curves at zero time. Data were fitted to the Michaelis–Menten equation for evaluation of the kinetic constants.

Although the pyrrolines do not appear to be substrates for MAO A, we examined their effects on 5-hydroxytryptamine (5-HT) metabolism in rat liver mitochondria. Six concentrations in triplicate (0, 0.01, 0.05, 0.1, 0.5 and 1 mM) of each compound were incubated for 15 min with 1 mg of mitochondrial protein at 30 °C in phosphate buffer (1 ml). Samples were then assayed with 5-HT (0.5 mM) using a radioassay method [9]. The I_{50} value for each pyrroline under these conditions was estimated from graphs of these data. The nature of the inhibitory process was not investigated further.

Investigation of the possible metabolites

Samples of mitochondrial protein (300 μ g) were incubated at 30 °C in 1 ml of phosphate buffer, pH 7.5, with either 1-methyl-3-phenyl- Δ^3 -pyrroline or 1-methyl-3-(4-chlorophenyl)- Δ^3 -pyrroline (0.5 mM) for 1 h. They were then extracted with diethyl ether (3 $\times$ 1 ml). As a control, mitochondria without added pyrroline were treated in an identical manner. The extracts were dried, taken up into 100 μ l of a mixture of HPLC solvents A and B (see below) and a 50 μ l sample was chromatographed on a Waters C₁₈ semi-preparative HPLC column using a Shimadzu instrument. Conditions: solvent A, 0.1 % trifluoroacetic acid (TFA) in water; solvent B, 0.1 % TFA in acetonitrile; gradient 20–80 % B over 30 min; flow rate 1.5 ml $\cdot$ min⁻¹.

Figure 3 Plots of v against $[S]$ for pyrrolines in presence of $300 \mu\text{g}$ of mitochondrial protein

The upper trace is the 3-phenyl derivative (I), the lower trace is the 3-(4-chlorophenyl) analogue (II). Points are mean values from triplicate assays. Conditions: buffer, 100 mM phosphate, pH 7.5; temperature, 30°C ; total assay volume, 1 ml; pathlength, 1 cm.

RESULTS

The spectral scans produced when the pyrroline derivative was incubated with mitochondria are shown in Figure 2. Computation of the data for v against $[S]$ for the two pyrrolines (Figure 3) gave the following K_m and V values respectively for pyrrolines (I) and (II): 0.079 mM , $0.113 \text{ A units} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of mitochondrial protein; 0.067 mM , $0.093 \text{ A units} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. If it is assumed that the product in each case is the corresponding pyrrole (see below), the $\Delta\epsilon$ values for reactions with (I) and (II) at the indicated wavelengths were calculated as $14000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (I) and $9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (II). These then translate to respective V_{max} values of 8.1 and $10.3 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. In the inhibition experiments with 5-HT as substrate, I_{50} values of 0.24 mM and 0.075 mM were obtained respectively for the phenyl- and chlorophenyl-pyrrolines.

Figure 4 shows typical progress curves obtained by continuous monitoring when the pyrrolines were incubated with mitochondria. Plots of time against $\ln(\text{activity})$ data obtained from these curves indicate a pseudo-first-order process in each case, with half-life values respectively for (I) and (II) of 10 min and 7.3 min. Data obtained from measurements of MAO activity [substrate (*E*)-cinnamylamine] after incubation with the pyrrolines and subsequent washing of the mitochondria are shown in Table 1.

HPLC traces of the diethyl ether extracts are shown in Figures 1(c) and 1(d). The peak at 17.14 min (Figure 1c) is absent from the control sample (no added pyrroline) (Figure 1d) and is well

Figure 4 Progress curves obtained by incubation of 0.5 mM pyrroline derivatives with $300 \mu\text{g}$ of mitochondrial protein

Top panel, 3-phenyl derivative (I); lower panel, 3-(4-chlorophenyl) analogue (II). Insets are plots of $\ln(\text{activity})$ against time. Conditions: buffer, 100 mM phosphate, pH 7.5; temperature, 30°C ; total assay volume, 1 ml; pathlength, 1 cm.

Table 1 Effect of preincubation of arylpyrrolines (I) and (II) (0.5 mM) on MAO activity

Mitochondria ($300 \mu\text{g}$ of protein) were incubated at 30°C , pH 7.5, for 35 min with (II), for 1 h (I) or for 3 h (both I and II). Samples were washed and centrifuged (three times) as described in the text before measuring MAO activity using (*E*)-cinnamylamine as substrate. Activity is expressed as nmol of (*E*)-cinnamaldehyde produced/min. All incubations were done in triplicate. Separate controls were run for each pyrroline. Control values at zero time refer to baseline enzyme activity measured without any pretreatment. Values in parentheses are percentage inhibition.

Incubation time (min)	MAO activity ($\text{nmol} \cdot \text{min}^{-1}$) (mean $\pm$ S.D.)			
	Control	Pyrroline (I)	Control	Pyrroline (II)
0		4.15 ± 0.38	4.48 ± 0.21	
35			4.62 ± 0.36	1.53 ± 0.09 (67)
60	4.42 ± 0.07	2.79 ± 0.31 (37)		
180	4.76 ± 0.34	1.16 ± 0.2 (76)	4.49 ± 0.27	0.5 ± 0.14 (89)

separated from the pyrroline itself (10.05 min). As can be seen from the overlay (Figure 1c), this substance has the same retention time as 1-methyl-3-(4-chlorophenyl)pyrrole. The same material

was also extracted from the reaction mixtures after acidification with HCl, reinforcing the view that it is the pyrrole, since pyrroles are not protonated under these conditions. In the case of the 1-methyl-3-phenylpyrroline, similar ether extracts produced a compound with retention time identical with that of authentic 3-phenyl pyrrole.

DISCUSSION

The data in Figure 2 show that a spectral change occurs when the pyrrolines are incubated with mitochondria, but not if they have been first incubated with the MAO B inhibitor (*R*)-deprenyl, indicating that this change is due to the action of MAO B. The MAO A inhibitor clorgyline was without effect on these spectral changes (results not shown), but the studies with 5-HT as substrate showed clearly that although the pyrrolines are not metabolized by MAO A, they interact with the enzyme to inhibit 5-HT metabolism. The non-linear progress curve obtained at the wavelengths indicated in Figure 4, when the pyrrolines were incubated with mitochondria, suggests that inhibition of the MAO B occurs concomitantly with, or as a consequence of, the oxidation. Data in Table 1 show that the recovery of enzyme activity was partly determined by the preincubation time of mitochondria with the pyrroline. In each case, progress curves showed that MAO B activity was almost totally inhibited by preincubation with the pyrrolines for 35 min (II) or 1 h (I), but partial recovery of activity was then observed after washing/centrifugation of the protein pellets. However, when preincubation was extended to 3 h, recovery of activity was substantially less, suggesting that a slower, irreversible process is also occurring.

It is believed that the oxidation of amines $RCH_2N(R^1)(R^2)$ by MAO proceeds by two one-electron transfers to flavin, with formation of an imine intermediate by loss of a proton from the α -carbon of the substrate. The imine is subsequently hydrolysed to $RCHO$ and $HN(R^1)(R^2)$. In the case of the cyclic amine MPTP, the cyclic imine 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) is the observed product. If the oxidation of 3-aryl- Δ^3 -pyrrolines proceeds in a similar manner, formation of the corresponding pyrrolinium cation would be expected, leading to a reaction such as suggested in Scheme 1. This ion could lose a proton to generate the corresponding pyrrole or be trapped by, e.g., water, or by a nucleophilic species on the enzyme. The results support such a scheme, since what appears to be a pyrrole is formed during the enzymic reaction and the MAO becomes inactivated. However, the data in Table 1 suggest that, after the initial reversible (or partially reversible) total inhibition occurs (Figure 4), a slower inactivation process takes place. A possible explanation is that a tightly bound, but dissociable, intermediate product slowly forms a covalent bond with the enzyme.

Ottoboni et al. [12] reported that, when ²H-labelled forms of MPTP were oxidized by MAO, the observed isotope effects suggested that proton loss is from the methylene C atom of the allylic structure (C-2 in the hetero ring). In the case of the pyrroline ring, the situation is more complex, because there are

two such carbon atoms present (C-2 and C-5). At present we do not know if involvement in the enzymic oxidation process is confined to just one of these two positions or whether either can be a participant, though highest-occupied-molecular-orbital ('HOMO') calculations [6] suggest that attack at C5 is more likely. In either case, the end product is likely to be the pyrrole.

The question arises of whether oxidation products of these pyrrolines might interfere with the electron-transport chain in the way that the MPTP metabolite MPP⁺ is found to do. The pyrrole may not behave like MPP⁺, since it would not be protonated under physiological conditions and the positive charge on the MPP⁺ appears to be important for its effects in intact mitochondria [13]. The intermediate pyrrolinium ion, analogous to MPP⁺, that we suggest may be derived from the pyrroline, would be expected to have a half-life notably shorter than that of MPP⁺ and might not therefore reach the inner membrane of mitochondria. Nevertheless, since some pyrrolines are likely to be present in some foods [14], and may be under consideration as additives in others [15], it will be important to examine the effects of these compounds on mitochondrial respiration. The recent report that MAO B of bovine liver is able to metabolize pyrrolines [6] does not say whether that enzyme is subject to inactivation by these compounds. It has been observed elsewhere [16] that rat and bovine MAO can show marked differences in sensitivity to some inhibitors, so it will be of interest to see if this is reflected in the effects of these pyrrolines. Human MAO is very similar in properties to the enzyme from rat, so it is likely that the effects reported here are likely to extend to human mitochondrial MAO B.

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