



0024-3205-(94)E0049-W

4-DIMETHYLAMINOPHENETHYLAMINE, A SENSITIVE, SPECIFIC, ELECTROCHEMICALLY DETECTABLE MONOAMINE OXIDASE-B SUBSTRATE

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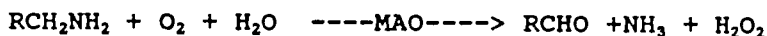
(Received in final form April 4, 1994)

Summary

4-Dimethylaminophenethylamine (DMAPEA) was characterized as an MAO substrate. This compound was unaffected by MAO-A, while its oxidation by MAO-B was linear as a function of both time and enzyme concentration, with $K_m = 5.8 \mu M$ and $V_{max} = 21.2 \text{ pmol/min/mg protein}$, using a crude rat brain mitochondrial suspension as source of MAO. Both DMAPEA and its oxidation product, 4-dimethylaminophenylacetic acid (DMPAA), can be detected electrochemically at 0.85 V. The high MAO-B affinity and selectivity of DMAPEA, together with its low oxidation potential, make this molecule a unique tool to determine MAO-B activity in a wide variety of tissue preparations using HPLC-ED.

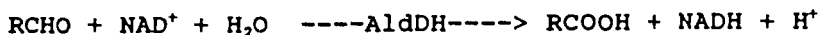
Key Words: MAO-B substrate, 4-dimethylaminophenethylamine, phenethylamines

Monoamine oxidase (EC 1.4.3.4, amine: O_2 oxidoreductase, MAO) catalyzes the oxidative deamination of endogenous aromatic monoamines, such as catecholamines, serotonin (5-HT) and trace amines such as β -phenylethylamine (PEA), octopamine and tyramine. Bioactive exogenous amines such as benzylamine are also metabolized by MAO [1]. The MAO-catalyzed oxidative deamination of primary monoamines to give the corresponding aldehydes is represented by the following equation:



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Generally, this metabolic pathway continues with a coupled reaction catalyzed by aldehyde dehydrogenase (AldDH) to yield the stable acidic metabolite:



There exist two types of MAO (MAO-A and -B) classified according to their substrate preference and sensitivity towards selective inhibitors. MAO-A selectively oxidizes 5-HT and is selectively and irreversibly inhibited by low concentrations of clorgyline. MAO-B selectively oxidizes benzylamine and low concentrations of PEA and is inhibited by 1-deprenyl [2,3].

Measurement of MAO activity from different tissues has clinical importance because disturbances in the activity of the enzyme have been, either directly or as clinical markers, related to different pathologies of the CNS such as affective disorders, schizophrenia or dementia [see for example 4,5,6].

Furthermore, the realization of the great therapeutic potential of selective MAO inhibitors (MAOI) as antidepressants [for a review see 7] and in the treatment of degenerative pathologies such as Parkinson's [8,9,10] and Alzheimer's diseases [8,11] requires sensitive and selective methods to measure different MAO activities.

There exist several methods to determine MAO activity [for reviews see 12,13], most of them based on the determination of some of the reaction products described above. Thus, disappearance of substrate [14] or ammonia generation [15] have been used to measure MAO activity. Determination of products from coupled reactions of H_2O_2 by spectrophotometry [16], fluorometry [17], and luminometry [18] have been employed. Aldehyde products can be directly measured spectrophotometrically [19] or, after derivatization, by fluorometry [20]. Acidic metabolites such as 5-hydroxyindoleacetic acid can be detected fluorometrically.

All these methods have distinct limitations, mainly due to their lack of sensitivity or selectivity. For this reason, the method most commonly employed to determine MAO activity is the radiochemical assay described by Wurtman and Axelrod [21] with a number of modifications [see for example 22,23].

An alternative method involving high-performance liquid chromatography (HPLC) techniques to separate products, associated with either ultraviolet or electrochemical (ED) detection, has been described [24,25]. In the case of the HPLC-ED method, the lack of selective and electrochemically detectable MAO-B substrates has limited its use.

3-Methoxy-4-hydroxybenzylamine (MHBA) has been proposed and used as a selective and electrochemically detectable MAO-B substrate [26]. Recently, an exhaustive optimization of the method employing this substance was reported [27]. Nevertheless, as can be inferred from this last publication, the selectivity of MHBA leaves much to be desired.

In a screening of some MAOI derivatives of 4-dimethylamino phenethylamine (DMAPEA), we recently reported that the parent compound of the series, i.e. DMAPEA, is a potent inhibitor of both

MAO isoforms with approx. $I_{50} = 20 \mu\text{M}$ for MAO-A and $10 \mu\text{M}$ for MAO-B [28]. In that work we pointed out that DMAPEA was metabolized during incubation with a crude rat brain mitochondrial preparation. Moreover, we speculated that the MAO inhibition profile exhibited by this drug could be due to its action as a competing substrate. On the other hand, we have shown that various 4-methylaminophenethylamine derivatives can be oxidized electrochemically at potentials below 0.85 V [29].

Considering these results, we have now investigated the possibility that DMAPEA is a MAO substrate and that this metabolism can be attributed specifically to MAO-B. In addition, we have examined the ability of DMAPEA and its MAO metabolites to be detected under standard conditions used for HPLC analysis of other MAO substrates such as catecholamines and serotonin, and their metabolites.

Materials and methods

Compounds

4-Dimethylaminophenethylamine (DMAPEA) acid tartrate was synthesized following a published sequence [30,31]. (-) Deprenyl was kindly provided by Prof. J. Knoll. THF and acetonitrile (ACN) were Merck HPLC grade. Clorgyline was from Research Biochemicals and 4-dimethylaminophenylacetic acid (DMPAA) was from Aldrich. Aldehyde dehydrogenase from yeast (AldDH) and β -nicotinamide-adenine dinucleotide (β -NAD) were from Sigma.

Quantitation of DMAPEA and its main putative MAO metabolite DMPAA

In all cases the products were determined by HPLC-ED. A C_{18} reverse phase column (ODS 250 mm x 4.6 mm, 5 μm , BIOPHASE, USA), an amperometric detector (BAS LC-3A) equipped with a glassy carbon electrode and a two-channel graphic recorder (BAS), were used to analyze the reaction mixtures. The mobile phase flow rate was 1 ml/min, and its composition was 31.5 g citric acid; 956 ml bidistilled water; 12 N NaOH to pH = 3; 200 mg sodium octyl sulfate; 40 ml ACN, and 50 ml THF. Detector sensitivity was 10 nA, and the oxidation potential was fixed at 0.85 V using a glassy carbon working electrode versus an Ag/AgCl reference electrode.

Assay of MAO activity

A crude mitochondrial suspension was prepared from whole brain (after discarding the cerebellum) of male Instituto de Investigaciones Biológicas Clemente Estable rats weighing 200-240 g as described previously [28] and was incubated with DMAPEA. MAO activity was assayed by HPLC-ED, measuring the heights of the chromatographic peaks of DMAPEA and its main oxidative metabolite (DMPAA). Under the experimental conditions described, the detection limit of this assay was 2-5 pmol of DMPAA (signal: noise ratio of 2:1). Protein concentration was determined according to Lowry et al. [32].

In all enzymological assays the reaction mixture contained AldDH (0.8 units) and β -NAD (0.6 μmol) dissolved in 150 μl phosphate buffer. In a standard assay the final volume was 0.5 ml and the volume adjustments were made with 0.1 M phosphate buffer (pH = 7.4). The mixture was incubated for 5 min at 37 °C in a shaking water bath in open test tubes. The enzymatic reaction was stopped by the addition of 200 μl of 1 M HClO₄. The mixture was centrifuged at 15000

x g for 10 min at 4 °C, and 50 µl of supernatant were injected into the HPLC-ED system. In some kinetic measurements, volumes larger than 50 µl (100 - 200 µl) were used. No significant band spreading of the chromatographic peaks were noted with these injection sizes.

- Linearity between product formation and different enzyme concentrations or incubation times.

DMAPEA (5 µM final concentration) was incubated at 37 °C for 5 min with different concentrations of enzyme (1-5 mg protein). In other experiments, DMAPEA (5 µM final concentration) was incubated under the same conditions with mitochondrial suspension (250 µl containing 2.3-2.5 mg protein) for different times (2-7 min). Each experiment was performed in triplicate with each of three different mitochondrial suspensions.

- Effects of selective MAO inhibitors.

Mitochondrial suspension (2.5 mg protein) was preincubated at 37 °C for 30 min with different concentrations of either 1-deprenyl or clorgyline. Then DMAPEA dissolved in 25 µl of distilled water (5 µM final concentration) was added and the incubation was maintained for 5 min at 37 °C in a shaking water bath. Each experiment was performed in triplicate.

- Kinetic parameters of DMAPEA.

DMAPEA was used as the enzyme substrate at nine different concentrations between 50 nM and 50 µM. MAO-A and MAO-B activities were defined as activity in the presence of 1-deprenyl (10^{-6} M) or clorgyline (5×10^{-7} M), respectively. These concentrations of MAO inhibitors are selective and completely inhibitory for the appropriate form of MAO in rat brain homogenates [see for example 33,34].

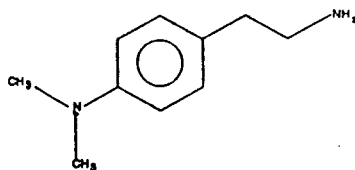
Mitochondrial suspensions (2.1-2.5 mg protein) were preincubated for 30 min with the inhibitors and then the substrate was added to different final concentrations, maintaining the incubation for 5 min at 37 °C in a shaking water bath. Km and Vmax values for DMAPEA were calculated by replotting the data collected from the experiments, as described by Segel [35]. Each experiment was performed in triplicate with each of four different mitochondrial suspensions.

All the results are presented as mean $\pm$ s.e.m.

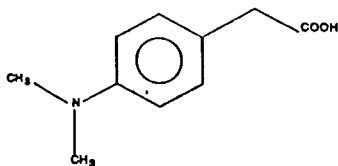
Results

Fig. 1 shows the chemical structures of DMAPEA and DMAPAA.

Fig.2 illustrates the chromatograms obtained for DMAPEA and its oxidation metabolites under different conditions. The peak shapes and retention times for DMAPEA and DMAPAA standards are shown in Fig. 2A. After incubating DMAPEA (5µM) with mitochondrial suspension during 5 min, three peaks could be detected in the supernatant of the reaction mixture, two of which had exactly the same retention times as the standards (Fig. 2B). When the substrate was incubated with mitochondrial suspension in the presence of AldDH (0.8 units) and β -NAD (0.6 µmol), only the two peaks with the same retention times as the standards could be detected (Fig. 2C). The effects of selective MAOIs 1-deprenyl (50 nM MAOI-B) and clorgyline (1 µM MAOI-A) are shown in Figs. 2D and 2E, respectively. Based on the



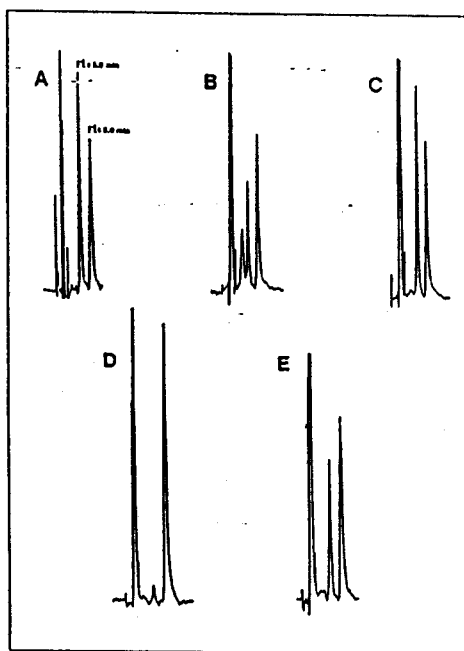
DMAPEA



DMPAA

Fig. 1

Chemical structures of DMAPEA and DMAPAA.

**Fig. 2**

Typical HPLC-ED chromatograms obtained for DMAPEA and its metabolites under different conditions. A) DMAPEA and DMAPAA standards. B) DMAPEA + mitochondrial suspension. C) DMAPEA + mitochondrial suspension + AldDH + β -NAD. D) DMAPEA + mitochondrial suspension + l-deprenyl (50 nM) + AldDH + β -NAD. E) DMAPEA + mitochondrial suspension + clorgyline (1 μ M) + AldDH + β -NAD. r.t. = retention time. For experimental details see text.

effects of these selective inhibitors (Figs. 2D-2E), MAO-B appears to be the main enzyme form implicated in the deamination of DMAPEA. The smaller peak appearing in Fig. 2D corresponds to the acidic metabolite of DMAPEA and is a consequence of the concentration of 1-deprenyl which is not enough to inhibit completely MAO-B. In all experiments the rate of product formation was paralleled to that of substrate disappearance.

Product formation was linear with both the incubation period (Fig. 3A) and the amount of protein (Fig. 3B).

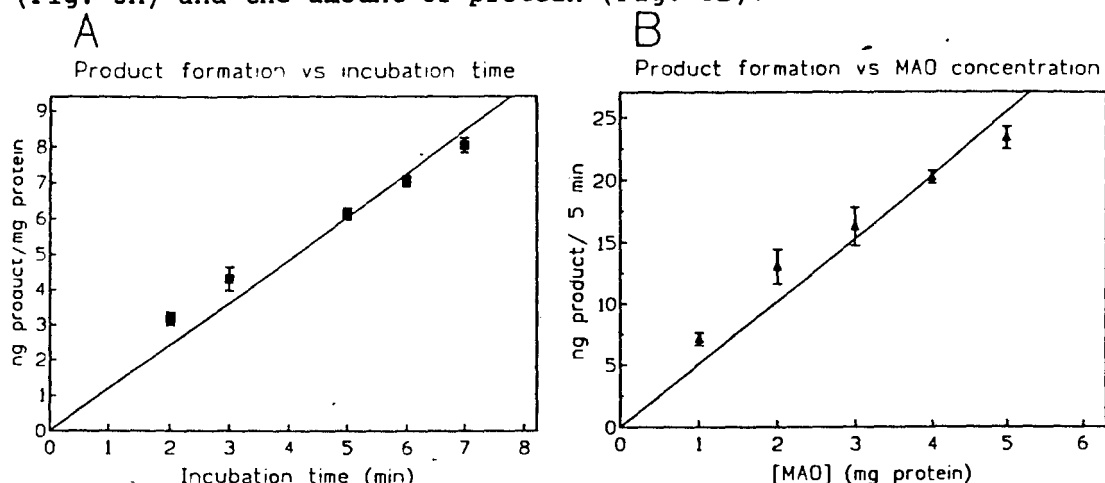


Fig. 3

Product formation as a function of incubation time (A) and amount of protein (B). Each point represents the mean $\pm$ s.e.m. of 9 determinations.

The kinetic constants for the oxidation of DMAPEA by the MAO-B present in crude rat brain mitochondrial suspension were: $K_m = 5,8 \pm 0,1 \mu M$ and $V_{max} = 21,23 \pm 0,16 \text{ pmol/min/mg protein}$. Oxidation of DMAPEA by MAO-A was not apparent or was less than the detection limit within the substrate concentration range assayed (5 nM - 50 μM). The K_m value for DMAPEA oxidation by MAO-B is much lower than currently available alternatives [36].

The inhibition patterns of DMAPEA deamination by 1-deprenyl or clorgyline are illustrated in Fig 4.

As with other MAO-B selective substrates such as PEA or benzylamine, DMAPEA deamination exhibited high sensitivity towards 1-deprenyl and low sensitivity towards clorgyline. The effect of clorgyline on DMAPEA oxidation is due to the fact that this drug inhibits MAO-B activity at more than micromolar concentrations.

Discussion

According to the results shown above, DMAPEA is clearly deaminated by MAO. The chromatographic peak of one of the products formed after incubation of this substance with a mitochondrial suspension is identical to the peak of the expected DMAPEA oxidation product, DMAPAA. When AldDH and its cofactor β -NAD are added to the

Effect of selective MAO inhibitors

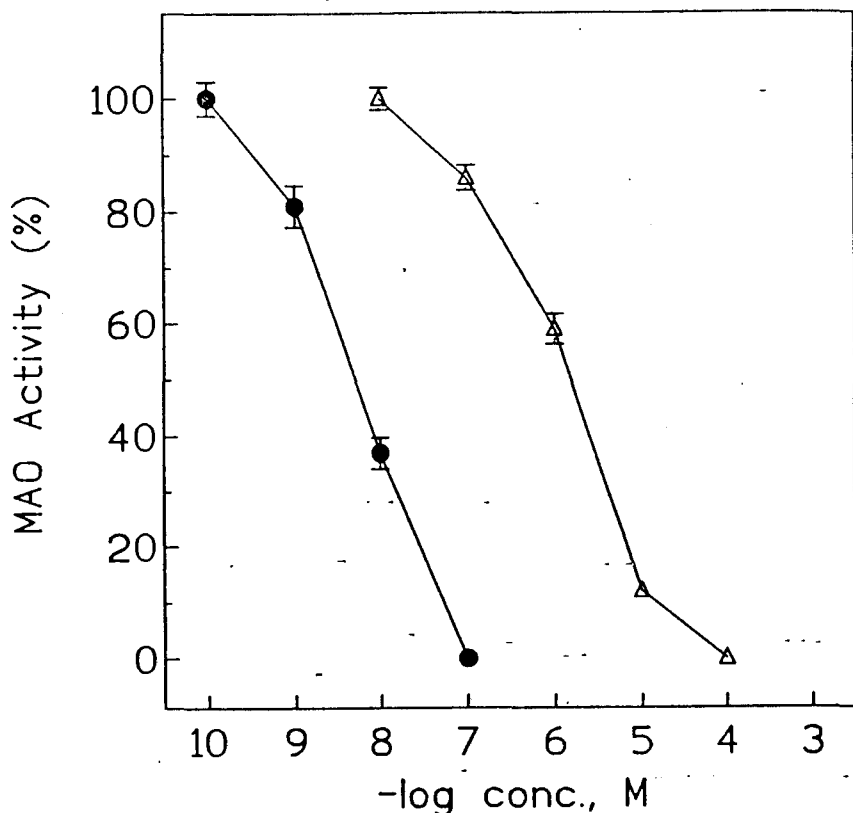


Fig. 4

Effects of selective MAO inhibitors 1-deprenyl (●) and clorgyline (Δ) on the MAO deamination of DMAPEA. The mitochondrial suspensions were preincubated for 30 min with inhibitors. The concentrations of inhibitors correspond to the final concentration after the addition of substrate dissolved in 25 μ l. Values are given as mean $\pm$ s.e.m. of triplicate experiments.

incubation medium, this peak increases at the expense of another, unidentified substance, presumably the aldehydic MAO metabolite. These results strongly suggest that DMAPEA is metabolized by MAO to the corresponding aldehyde which is then transformed into the acidic metabolite by AldDH present in the medium.

Furthermore, DMAPEA is a specific MAO-B substrate with a unique biochemical profile. Its specificity is not based on different affinities for both MAO isoforms, like other selective substrates including MHBA [27], but on different pharmacological properties of the molecule. Thus, DMAPEA is only metabolized by MAO-B, and inhibits MAO-A.

Based on the fact that several phenethylamines are MAO substrates, the fundamental activity of DMAPEA towards oxidation by

MAO is afforded by the underlying phenethylamine structural feature. The affinity of this molecule for MAO-A may be related to the presence of the 4-dimethylamino group which is found in many selective MAO-A inhibitors [37,38].

MAO-A activity can easily be determined using serotonin as an electrochemically active, selective substrate, giving a metabolite which can be quantified simultaneously under the same conditions. The high selectivity and affinity of DMAPEA for MAO-B, as well as the electrochemical properties of both DMAPEA and its oxidation product DMAPAA, make this substrate a useful tool to determine MAO-B activity in a wide variety of tissue preparations using an inexpensive, rapid and very sensitive method.

Acknowledgements

This work was partially supported by International Program in Chemical Sciences (IPICS), Programa de Desarrollo de las Ciencias Básicas (PEDECIBA), Latin American Network for Research on Bioactive Natural Products (LANBIO) and Fondo Nacional de Ciencia y Tecnología (FONDECYT grant n° 915/89). Authors thank Laura Flores and Ivana Faccini for their secretarial assistance.

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