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CONCENTRATION DEPENDENCE OF THE OXIDATION OF TYRAMINE BY THE TWO FORMS OF RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE

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Abstract—The two forms of monoamine oxidase in rat liver mitochondria were shown to have different K_m and maximum velocity values with tyramine as the substrate. K_m values of 107 ± 15 μM and 579 ± 45 μM were determined for the A- and B-forms respectively at pH 7.2 in air-saturated buffer. The maximum velocity of the A-form was found to be approximately half of that of the B-form under these conditions. A consequence of these differences is that the ratio of activities of MAO-A:MAO-B determined from clorgyline inhibition curves will be dependent upon the concentration of tyramine used to assay for enzyme activity. At a fixed concentration of tyramine, the height of the plateau in a clorgyline inhibition curve will also be affected by the presence of a selective competitive inhibitor. Procaine, an MAO-A selective competitive inhibitor, was found to increase the K_m value of the MAO-A towards tyramine to 505 ± 172 μM and under these conditions the plateau-height of the clorgyline inhibition curve was not significantly affected by variations of the tyramine concentration over a 20-fold range.

Two forms of the enzyme monoamine oxidase (MAO) (amine:oxygen oxidoreductase (deaminating) (flavin-containing) EC 1.4.3.4) are present in many mammalian organs. One form, which has been termed the A-form, is sensitive to inhibition by low concentrations of clorgyline whereas the other, the B-form, is sensitive to inhibition by low concentrations of deprenyl [1, 2]. In rat liver, tyramine has been shown to be a substrate for both forms of the enzyme, whereas 5-hydroxytryptamine is metabolised predominantly by the A-form and 2-phenethylamine predominantly by the B-form of the enzyme [3; 4]. The substrate specificities of the two forms are, however, not the same in all tissues [5].

Recent work has suggested that differences in the K_m values of the two forms for their substrates may be important factors in their specificity differences. The K_m values of pig liver MAO-A towards 5-hydroxytryptamine has been reported to be ten times lower than that of the B-form of the enzyme for this substrate [6], and in the case of 2-phenethylamine the K_m value of the B-form of the enzyme from several species has been reported to be considerably lower than that of the A-form [see e.g. 7–9]. The proportions of the two forms that are present in tissues are frequently assessed by determining their response to selective irreversible inhibitors at a single concentration of tyramine and any difference between the K_m values of the two forms for this substrate would cause the results obtained to be substrate concentration dependent. There have, however, been no detailed studies on the K_m values of the two forms for tyramine. In this paper we report the results of such a study and consider its inplications for the use of selective irreversible inhibitors in the determination of the proportions of the two forms that are present.

MATERIALS AND METHODS

Male Wistar rats of body weight 250–300 g were killed by a blow to the head and the livers were rapidly removed, blotted on filter paper and weighed. All subsequent procedures were performed at 0–4°. The livers were homogenised in five vol. (w/v) of 0.25 M sucrose, 10 mM potassium phosphate, pH 7.2, using a Dounce homogeniser. The homogenates derived from pairs of rats were pooled and centrifuged at 600 *g* for 10 min to remove unbroken cells, nuclei and cell debris. The supernatants were carefully decanted and centrifuged at 15,000 *g* for 10 min and the mitochondrial pellets obtained were resuspended in the sucrose–phosphate mixture and centrifuged again for 10 min at 15,000 *g*. The pellets were resuspended in the sucrose–phosphate mixture to a protein concentration of 5 mg·ml^{-1} and stored frozen at −20°.

Monoamine oxidase (MAO) activity was assayed radiochemically by a modification of the method of Otsuka and Kobayashi [10]. Assays were carried out in 7 ml plastic liquid scintillation mini-vials. A mixture containing 200 μl 0.1 M potassium phosphate buffer, pH 7.2, 100 μl of the mitochondrial suspension and 50 μl of water, clorgyline or deprenyl as appropriate was allowed to equilibrate with continuous shaking, at 37° for 30 or 60 min. 50 μl of ^{14}C-labelled tyramine was then added and incubation was continued for a predetermined time before the reaction was stopped by the addition of 100 μl of 2 M citric acid. Blank values were obtained by the addition of the citric acid before the substrate was added. Five ml of toluene:ethyl acetate (1:1 v/v)

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containing 0.6 percent (w/v) PPO (2,5-diphenyloxazole) was then added to each vial and they were capped and mixed three times on a vortex mixer. The vials were then placed in a freezer at −20° and left for at least 3 hr to allow the aqueous layer to freeze. The organic layer from each vial was then poured into a fresh 7 ml mini-scintillation vial and the radioactivity was determined in a Packard liquid scintillation counter. The values obtained were corrected for the efficiency of extraction of the deaminated metabolites into the organic layer [11] to allow activities to be expressed as nmoles substrate metabolised·mg protein^{-1}·min^{-1}. In all cases it was ensured that product formation was linear with time up to the time period used for the assay so that the values obtained corresponded to the initial velocities of the enzyme-catalysed reaction.

Protein concentration was measured by the method of Markwell *et al.* [12].

Tyramine-[7-^{14}C]-hydrochloride was obtained from the Radiochemical Centre, Amersham, U.K. Clorgyline hydrochloride was a gift from May & Baker Ltd., Dagenham, U.K. and *l*-deprenyl hydrochloride was a gift from Prof. J. Knoll, Semmelweis University of Medicine, Budapest, Hungary. All other materials were standard laboratory chemicals of analytical reagent grade where possible.

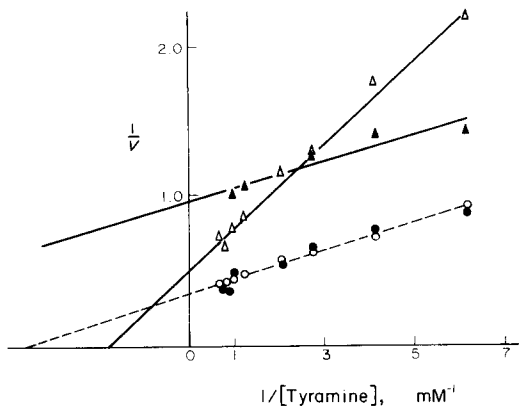

Fig. 1. Double-reciprocal plots of the activity of rat liver mitochondrial monoamine oxidase with tyramine as the substrate. Before assay, samples were preincubated for 60 min at 37° with distilled water (●), 0.3 μM *l*-deprenyl (▲) or 0.3 μM clorgyline (△). Data points represent the mean values of determinations made with three mitochondrial preparations and the lines were fitted by linear regression analysis. Data points (○) were calculated for the uninhibited enzyme from the individual K_m and V_{max} values (summarised in Table 1) for the clorgyline- and *l*-deprenyl-inhibited preparations.

RESULTS AND DISCUSSION

Clorgyline and deprenyl were used to inhibit either the A- or B-form of the enzyme so that the kinetic parameters of the other could be determined. These compounds react with the enzyme by first forming a non-covalent enzyme–inhibitor complex followed by irreversible reaction to form a stable covalent adduct within this complex [see 13–15]. Clorgyline forms this adduct relatively rapidly and after 20 min of preincubation of rat liver monoamine oxidase with low concentrations of this inhibitor irreversible reaction with the A-form of the enzyme has been shown to be essentially complete [16, 17]. The irreversible reaction between *l*-deprenyl and MAO-B is somewhat slower [15, 16]. Since the presence of reversible enzyme–inhibitor complexes in the reaction mixture would raise the observed K_m values of the remaining enzyme activity, a 60 min preincubation between inhibitor and the enzyme preparation was used to ensure that the irreversible reaction had gone to completion. Concentrations of 3×10^{-7} M clorgyline and *l*-deprenyl were used to inhibit the A- and B-forms of the enzyme, respectively. These concentrations were on the plateau regions of the biphasic inhibitor curves of tyramine oxidation by these inhibitors (see e.g. Fig. 2), and in consequence it can safely be assumed that these inhibitors result in the complete inhibition of one form of the enzyme without significantly affecting the activity of the other form. Such selectivity has been found with these inhibitors in previous studies in both rat liver [see, e.g., 11] and human brain [14].

Table 1. Kinetic parameters of the A- and B-forms of rat liver mitochondrial monoamine oxidase activity towards tyramine*

Inhibitor	Form remaining	K_m (μM)	V_{max} (nmoles·mg protein^{-1}·min^{-1})
None	A and B	313 ± 4	3.01 ± 0.16
l-Deprenyl	A	107 ± 15	1.06 ± 0.13
Clorgyline	B	579 ± 45	2.01 ± 0.16

* The mitochondrial preparations were incubated for 60 min at 37° with either distilled water or 0.3 μM of the appropriate inhibitor before activities were determined as described in the text. 6–8 substrate concentrations in the range 160–1440 μM were used in each case and results were plotted both as $1/v$ against $1/s$ and as s/v against s. K_m and V_{max} values were calculated from each plot by linear regression analysis and the values obtained from the two plots were averaged to compensate for their inherent bias towards low and high substrate concentrations. All values represent the means ± S.E.M. for determinations with three mitochondrial preparations. In an additional experiment in which a tyramine concentration range of 32–288 μM was used, K_m and V_{max} values of 136 ± 31 μM and 1.03 ± 0.27 nmoles·mg protein^{-1}·min^{-1}, respectively (mean ± S.E.M.) were calculated for the A-form.

The kinetic parameters of the uninhibited enzyme are compared with those of the A- and B-forms in Table 1 and the kinetic plots from which they were derived are shown in Fig. 1. The sum of the maximum velocity values of the two individual forms was equal to the value obtained for the uninhibited enzyme. The initial velocity behaviour of the uninhibited enzyme may be represented by the equation:

$$v = \frac{V^{A}_{max}s}{K^{A}_{m} + s} + \frac{V^{B}_{max}s}{K^{B}_{m} + s} \quad (1)$$

where v represents the initial velocity, s the tyramine concentration, V_{max} and K_m are the maximum veloc-

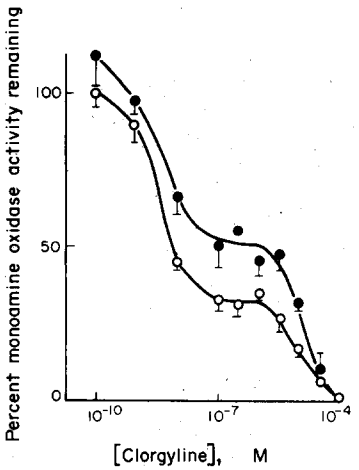

Fig. 2. Inhibition of rat liver mitochondrial monoamine oxidase by clorgyline. Samples were preincubated with the indicated concentration of clorgyline for 30 min at 37° before being assayed with either 50 μM (○) or 1.0 mM (●) tyramine. Each point represents the mean ± S.E.R. of duplicate determinations made with each of three separate mitochondrial preparations of the percentage of monoamine oxidase activity remaining with respect to samples preincubated for the same time with distilled water.

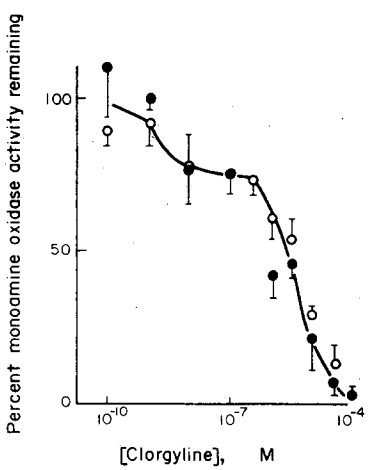

Fig. 3. Inhibition of rat liver mitochondrial monoamine oxidase by clorgyline in the presence of procaine. Samples were preincubated with the indicated concentrations of clorgyline in the presence of 30 μM procaine hydrochloride for 30 min at 37° and then assayed for activity with either 50 μM (○) or 1.0 mM (●) tyramine. Each point represents the mean ± S.E.R. of duplicate determinations made with each of three separate mitochondrial preparations of the percentage of activity remaining with respect to samples preincubated for the same time with 30 μM procaine hydrochloride.

ities and the Michaelis constants, respectively. The superscripts refer to the enzyme forms. Substitution of the parameters shown in Table 1 into this equation allowed the behaviour of a mixture of the two forms to be calculated and Fig. 1 shows that, when plotted in double-reciprocal form, the values calculated were in excellent agreement with those determined for the uninhibited enzyme.

Equation (1) may be expressed in reciprocal form as:

$$\frac{1}{v} = \frac{1 + (K_m^A + K_m^B)/s + K_m^A K_m^B/s^2}{V_{max}^A + V_{max}^B + (V_{max}^A K_m^B + V_{max}^B K_m^A)/s} \quad (2)$$

This predicts a non-linear dependence of $1/v$ on the reciprocal substrate concentration, but it can be calculated that with the values shown in Table 1 the degree of curvature will be relatively slight and would only become apparent over a greatly extended range of substrate concentrations.

The data in Table 1 can also be used to calculate the contributions each of the two forms would make to the total activity at different tyramine concentrations. Thus at a concentration of 50 μM tyramine, 32 ± 4 per cent of the total activity would be due to monoamine oxidase-B whereas at 1.0 mM tyramine this form would contribute 57 ± 2 per cent of the activity. Such changes in the ratio of the activities of the two forms at different tyramine concentrations were observed experimentally from clorgyline-inhibition curves (Fig. 2). Since the relative activities of the two forms that are present in monoamine oxidase preparations are frequently determined from the height of the plateau region in a clorgyline inhibition curve at a fixed, arbitrary, concentration of tyramine, the concentration-dependence reported here could account for some of the discrepancies in previously reported values.

Equations (1) and (2) show that the presence of a selective competitive inhibitor of MAO, which would cause an increase in the K_m value of the appropriate enzyme form, would affect the apparent relative activities of MAO-A and -B determined at any fixed non-saturating concentration of tyramine. Procaine hydrochloride has been shown to be a selective reversible inhibitor of the A-form of monoamine oxidase [18]. The presence of 30 μM procaine hydrochloride was found to increase the apparent K_m value of the A-form, measured after preincubation of the enzyme with 3×10^{-7} M deprenyl, to 505 ± 172 μM without significantly affecting the K_m value of the B-form (580 ± 40 μM). Thus, under these conditions, the K_m values of the two forms were not significantly different and this would be expected to result in the ratio of activities of the two forms revealed by clorgyline-inhibition curves being insensitive to variations in the concentration of tyramine. As shown in Fig. 3, this was found to be the case since no significant difference was observed in the plateau height of the clorgyline-inhibition curve at either 50 μM or 1 mM tyramine.

The data presented in this paper indicate that the K_m of the A-form of monoamine oxidase towards tyramine is significantly lower than that of the B-form. This difference would result in the proportions of the two forms revealed by clorgyline-inhibition curves being dependent on the concentration of tyramine used to assay for enzyme activity. The difference can be masked by the presence of a sufficient concentration of an MAO-A selective inhibitor, such as procaine, to render the apparent K_m values of the two forms equal. This effect may be of physiological importance since the presence of endogenous selective competitive inhibitors in tissue preparations

would be expected to result in changes in the apparent relative activities of the two forms.

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