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Proteases as Probes of Mitochondrial Monoamine Oxidase Topography *in Situ*

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SUMMARY

Selective inactivation of the multiple forms of mitochondrial monoamine oxidase (MAO) by proteases in intact and hypotonically disrupted rat liver mitochondria has been used to examine the question of differential membrane orientations of the A and B enzymes. Proteases used as probes included trypsin, β-chymotrypsin, and the extracellular protease of *Staphylococcus aureus*, chosen for their different amino acid specificities. With all three proteases, no changes in the relative rates of MAO-A and MAO-B inactivation were observed after disruption of the mitochondria. Trypsin and β-chymotrypsin gave much faster rates of MAO-A inactivation in both intact and disrupted mitochondria. The selective effect of trypsin on MAO-A was also confirmed in human placental mitochondria, which possess only A-type activity. The effectiveness of hypotonicity in disrupting the outer membrane of the mitochondria was shown by rapid protease inactivation of an intermembrane space marker enzyme, adenylate kinase (EC 2.7.4.3). Contrary to some recent reports in the literature, these findings strongly suggest that the MAO-A and MAO-B multiple-form catalytic activities do not reside on opposite faces of the membrane.

INTRODUCTION

Since the concept of multiple forms of mitochondrial MAO[1] emerged in the late 1960s to explain variations in substrate and inhibitor sensitivity among tissue sources, most biochemical research on this important enzyme has centered on the physical separation of the so-called A and B forms and the elucidation of structural features which may distinguish them. In 1973 it was suggested by Houslay and Tipton (1) that the A and B forms of this integral membrane protein may actually exist as a single-core peptide in two different local lipid environments. This proposal stimulated numerous subsequent studies attempting to define the role of bound lipid in MAO structure-activity relationships. Unfortunately, most of these investigations have either made use of solubilized enzyme preparations (2, 3) or examined the effect of various delipidation procedures on membrane-bound MAO (4–7), leaving unanswered the central question of the relationship of MAO structure to its native lipid environment.

An interesting exception is a study by Russell *et al.* (8) using immunological probes, which suggested that there were different membrane orientations for the A and B forms in intact mitochondria. Their methodology involved preparation of antisera to partially purified human brain and liver MAO which bound the antigens to give partial catalytic inhibition with both A and B substrates. Incubation of the antisera with intact mitochondria led to selective inhibition of MAO-B. However, when the mitochondria were hypotonically disrupted, both A and B activities were comparably inhibited. This led the authors to conclude that MAO-B activity was primarily located on the cytosolic face of the membrane whereas MAO-A was on the inner face.

A recent study by Huang (9) has shown that MAO-A was selectively inactivated by phospholipase C treatment, which also increased the fluidity of the central portion of the lipid bilayer. This, the author postulated, suggested that the A form was, at least, more deeply buried in the membrane, consistent with the thesis of Russell *et al.* (8).

A differential orientation of MAO-A and MAO-B activities within the bilayer would have important implications both in terms of a mechanism for MAO substrate selectivity and possible relationships to other mitochondrial functions, such as coupling of MAO oxygen utilization to respiratory components (10). We thought it of importance, therefore, to develop alternative methodologies to examine this question. Our approach has been

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[1] The abbreviations used are: MAO, monoamine oxidase; 5HT, 5-hydroxytryptamine (serotonin); PEA, β-phenylethylamine.

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to compare the rates of inactivation of MAO-A and MAO-B in intact and hypotonically disrupted mitochondrial preparations using a variety of proteolytic enzymes which have been shown not to penetrate the intact membrane (11). An asymmetrical distribution of MAO activities across the membrane would then be expected to result in different patterns of degradation with respect to metabolism of A and B substrates, 5HT and PEA, when intact and disrupted mitochondria are compared, analogous to the immunoinhibition study of Russell *et al.* (8).

Previously, a selective inactivation of MAO-A by trypsin was reported for solubilized, partially purified rat liver enzyme (3) and for a crude extract from rat cervical ganglia (12). Oreland and Ekstedt (13) found greatly increased sensitivity to trypsin for solubilized MAO and delipidated mitochondria relative to intact mitochondria isolated from pig liver. Weiss and McCauley (14) found no effect on either MAO-A or MAO-B in intact rat liver mitochondria and outer membrane preparations when treated with trypsin or the nonspecific protease mixture, pronase, at low concentrations for short periods of time.

For our methodology to reveal whether any of the MAO activity is inaccessible to proteoytic inactivation in intact mitochondria, the effect of the proteases themselves on outer membrane integrity must be much less than that of hypotonic disruption. Previous work has shown inner membrane markers of intact mitochondria to resist trypsin treatment (11). In the present study, the same marker enzyme for mitochondrial lysis was used as in the immunoinhibition experiments of Russell *et al.* (8). This is the enzyme adenylate kinase (EC 2.7.4.3), which is reported to be localized in the space between the outer and inner membranes and shows a high sensitivity to trypsin inactivation only under conditions resulting in rupture of the outer membrane (11).

Rat liver mitochondria with both A and B activities were used for most of these experiments. Substrate-selective effects of trypsin were also confirmed in a preparation of human placental mitochondria with only A-type activity. The proteolytic enzymes whose action on membrane-bound MAO have been examined include trypsin, β-chymotrypsin, and *Staphylococcus aureus* V8 protease, chosen for their different amino acid residue specificities (basic, neutral, and acidic).

EXPERIMENTAL PROCEDURES

Materials. Trypsin and β-chymotrypsin (bovine pancreas) and their peptide inhibitors (turkey egg white or soybean) were purchased from Sigma Chemical Company (St. Louis, Mo.). The *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories (Elkhart, Ind.). Substrates for MAO radioassays, [^{14}C]PEA and [^{14}C]5HT, were from New England Nuclear Corporation (Boston, Mass.). Reagents for the adenylate kinase assay, including hexokinase, glucose-6-phosphate dehydrogenase, ADP, and NADP, were from Sigma Chemical Company.

Isolation of mitochondria. Rat livers were obtained from young Sprague-Dawley rats within 10 min after decapitation. After removal of blood clots and connective tissue, the livers were minced, and a 25% homogenate was prepared in 0.25 M sucrose/10 mM potassium phosphate (pH 7.2)/0.5 mM EDTA by a minimal number of passes with a glass-Teflon homogenizer. Mitochondria were purified either by centrifugation in 0.25 M sucrose as described by Kearney *et al.* (15) or using a discontinuous Ficoll gradient (16). Both procedures gave mitochondria of similar MAO specific activities with PEA and 5HT as substrates (specific activity of PEA = 4.13–7.80 nmoles/min/mg; specific activity of 5HT = 2.72–4.10 nmoles/min/mg; PEA/5HT = 1.5–1.9).

Human placentas were obtained from the UCLA Obstetrics-Gynecology Department and placed in ice immediately following delivery. Isolation of mitochondria was commenced within 2 hr. The umbilical cord and amnionic membrane were removed, and the tissue was washed thoroughly in isotonic medium to remove blood. Mitochondria were purified from a 25% homogenate by centrifugation in 0.25 M sucrose as described for the rat liver preparation. Specific activities were 7.0 nmoles/min/mg with PEA as a substrate and 12.5 nmoles/min/mg with 5HT (PEA/5HT = 0.56).

Intactness of mitochondria in isotonic media was checked by electron microscopy and trypsin sensitivity of the marker enzyme, adenylate kinase, as mentioned under Introduction. This assay was carried out with the 1:5 ratio of trypsin to mitochondrial protein used in our MAO experiments. The assay procedure was that of Kuylenstierna *et al.* (11). By the biochemical criteria, our mitochondrial preparations, when stored at 0° in 0.25 M sucrose/10 mM potassium phosphate (pH 7.4) (protein 40 mg/ml), were more than 80–90% intact initially and began to show a significant further loss of integrity after about 5 days of storage.

Hypotonic disruption of mitochondria and treatment with peptidases. Stock suspensions of mitochondria (40 mg/ml protein) in 0.25 M sucrose/10 mM phosphate (pH 7.4) were diluted 1:10 with either 0.25 M sucrose or water and allowed to stand for 15 min at room temperature before initiation of the digestion by addition of a stock peptidase solution. Incubations were carried out in a shaker-water bath at 30° with trypsin and 37° with the less reactive β-chymotrypsin and *S. aureus* V8 protease. Aliquots were removed for MAO assay at the time intervals shown in the appropriate figures. For trypsin and β-chymotrypsin, the reaction was terminated by the addition of either soybean or turkey egg white trypsin inhibitor from stock solutions at a 1:1 weight ratio with respect to the amount of protease present. Zero-time controls for loss of MAO activity contained appropriate amounts of protease and inhibitor added simultaneously. In the case of the *S. aureus* protease, the inhibitor for this enzyme, diisopropyl fluorophosphate (17), was found to inhibit directly MAO-A selectively at concentrations required to inhibit the protease. As this digestion proceeded quite slowly, it was found sufficient to cool aliquots for MAO assay to 0° to terminate the reaction.

MAO assay. MAO was assayed with ^{14}C-labeled PEA and 5HT at 200 μM concentration for 15 min at 30° using the method of Wurtman and Axelrod (18). The PEA assay mixture was extracted with toluene and that with 5HT using 1:1 ethyl acetate-benzene to separate products. As PEA has been reported to show lower specificity for MAO-B with increasing concentration (19), selectivity of clorgyline and deprenyl inhibition was checked at the 200 μM substrate concentration used in these experiments. Inhibition by 5 μM deprenyl was 94% and that by 0.1 μM clorgyline was only 15%, indicating that a high degree of MAO-B selectivity is maintained at the PEA levels used in these experiments.

RESULTS

In Fig. 1 is shown a comparison of the rates of loss of MAO activity with PEA and 5HT as substrates for the intact and disrupted rat liver mitochondria at a 1:5 ratio of trypsin to total mitochondrial protein. The A form of the enzyme was much more sensitive to trypsin, consistent with earlier studies on soluble rat liver MAO and a crude tissue extract (3, 12). The small decrease in PEA activity (about 10% in 2 hr) may be due, at least in part, to that portion of PEA metabolized by the A form, since, as previously noted, this substrate becomes less selective at higher concentrations (19). The most important observation from this experiment is that there was no

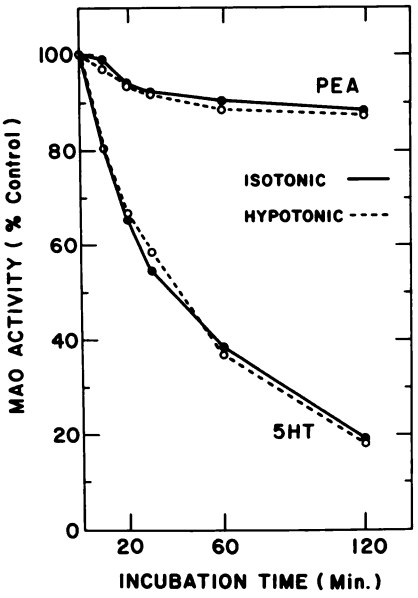

FIG. 1. *Inactivation of MAO in intact and hypotonically disrupted rat liver mitochondria by trypsin*

The trypsin to protein ratio was 1:5. The incubation temperature was 30°. Data points represent the means of three experiments.

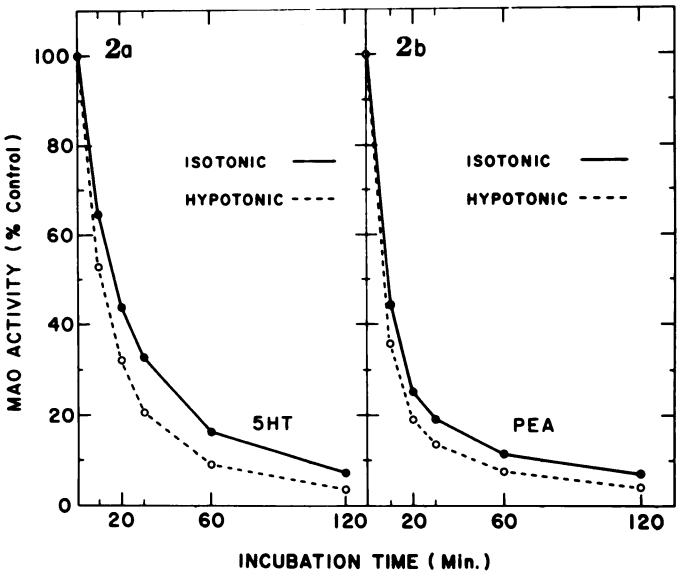

FIG. 2. *Same as in Fig. 1 except that human placental mitochondria was used as the tissue source*

The trypsin to protein ratio was 1:20. Data points represent means of two experiments.

significant change in the rate of inactivation for either substrate following hypotonic disruption of the mitochondria.

The specificity of trypsin degradation for MAO-A was also confirmed by examining its effect on MAO activity in mitochondria isolated from human placenta. PEA, normally considered to be an MAO-B specific substrate, is quite reactive with the MAO of this tissue, with a K_m comparable to that for 5HT (20). Human placenta, nevertheless, has been proposed to have only A-type MAO on the basis of clorgyline and deprenyl inhibitor sensitivities (20, 21).

Consistent with the notion of a single MAO species in this tissue, and contrary to our results with rat liver, the placental mitochondrial activities with either substrate showed a parallel pattern of rapid decay, the loss of PEA activity being actually somewhat greater up to 60 min of digestion (Fig. 2). The 4-fold lower concentration of trypsin required for comparable rates of MAO inactivation indicates increased sensitivity with respect to both substrates in this tissue. A somewhat faster loss of activity was observed after hypotonic disruption; however, there was no significant difference between the two substrates in this regard.

When the effect of β-chymotrypsin on rat liver mitochondrial MAO was examined, it was found that more vigorous conditions were required for appreciable inactivation rates (1:1 protease-protein ratios, 37° incubation). The results of these experiments, presented in Fig. 3, show a somewhat greater divergence between the intact and disrupted mitochondria and a smaller difference between inactivation rates for the two substrates, but otherwise the same pattern as that observed with trypsin. Again, the rate of loss of 5HT activity was greater and hypotonic disruption of the mitochondria resulted in very similar increases in the rate of inactivation for both substrates, indicating no selective exposure of new sites to cleavage for either form.

The third protease whose effect on membrane-bound MAO has been investigated in this study is the bacterial enzyme isolated from *Staphylococcus aureus* V8, which has been shown to be highly selective for cleavage at only aspartate and glutamate peptide linkages (17). As might be expected from this high degree of specificity, this protease inactivates MAO very slowly even at 37° (1:5 protease:protein ratio). For unknown reasons, this reaction also showed much more variability from one run to another than seen with the other proteases. As apparent differences in rates of MAO inactivation between dis-

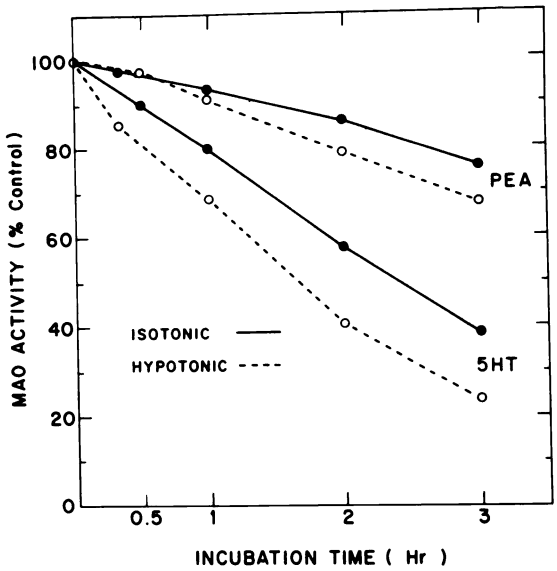

FIG. 3. *Same as in Fig. 1 except that the protease used was β-chymotrypsin*

The peptidase to protein ratio was 1:1. The temperature was 37°. Data points represent the means of five experiments.

rupted and intact mitochondria and between the substrates PEA and 5HT were quite small, the data from four experiments were analyzed statistically. A two-tailed *t*-test indicated no statistical significance to this apparent greater rate of MAO-A inactivation or for the differences between isotonic and hypotonic preparations (Fig. 4).

Figure 5 shows the time course for inactivation of adenylate kinase by trypsin and chymotrypsin, as a marker for disruption of the rat liver mitochondrial outer membrane in isotonic and hypotonic media. Mitochondrial preparations and protease concentrations were the same as those used in the MAO inactivation experiments. As indicated in Fig. 5, both proteases completely destroyed the adenylate kinase in hypotonic medium in less than 5 min (minimum incubation time before the first assay). In contrast (Figs. 1 and 3) after a 10-min exposure to trypsin under the same conditions, loss of MAO-A activity was 20% and that of MAO-B 4%, whereas in the presence of chymotrypsin there was less than 5% inactivation for either form. In isotonic medium the adenylate kinase was also slowly inactivated, suggesting increasing permeability of the outer membrane to proteins over the period of incubation with the proteases. Unlike the situation with MAO, where very different rates of inactivation were observed between trypsin and chymotrypsin, both proteases attacked adenylate kinase at similar rates: 60% of the activity was destroyed in the 1st hr by trypsin and 56% by chymotrypsin.

Adenylate kinase has been reported to be released from hyptonically disrupted mitochondria in the presence of EDTA (22). Release of the enzyme from the mitochondria prior to attack by the proteases would obviously effect arguments about their ability to cross the outer membrane—a premise on which our conclusions in regard to the location of the MAO-A and MAO-B forms is predicated [as well as those of Russell *et al.* (8)]. To determine the portion of adenylate kinase remaining associated with the mitochondria following exposure to hypotonic media, mitochondria diluted 10:1 in water were centrifuged at 40,000 × *g* for 20 min and reassayed for

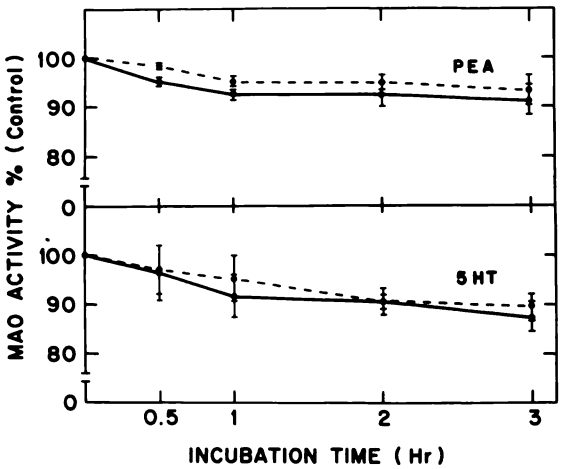

FIG. 4. *Same as in Fig. 1 except that the protease used was derived from Staphylococcus aureus V8*

The temperature was 37°. Data points represent the means of four experiments.

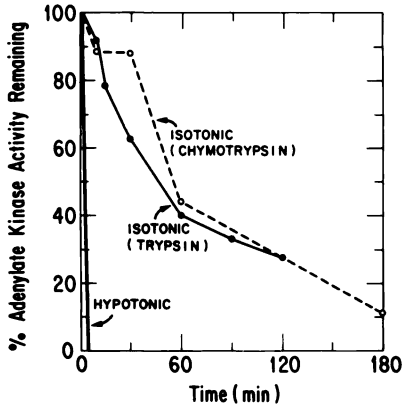

FIG. 5. *Inactivation of adenylate kinase in intact and hypotonically disrupted rat liver mitochondria by trypsin (——) and chymotrypsin (- - -)*

Same conditions as for MAO inactivation (Figs. 1 and 3).

adenylate kinase. The resuspended mitochondrial pellet after centrifugation was found to retain 29–65% of the original adenylate kinase activity.

Electron micrographs of rat liver mitochondria in isotonic medium showed well-formed, normal-appearing organelles with a high degree of homogeneity (~85%).

The hypotonically treated preparations revealed no obvious discontinuities in the membrane at ×41,000 magnification. The majority of the mitochondria appeared as whole organelles, with the outer membrane pulled away from the matrix, and showed little of the cristae detail. The remaining structures appeared to consist of only membrane vesicles or matrix material. These were estimated to be in ratios of 1:0.27:0.77.

DISCUSSION

The absence of any significant effect of hypotonic disruption of the rat liver mitochondria on the relative rates of inactivation of MAO-A and MAO-B by trypsin, together with the much faster inactivation of MAO-A, argues strongly against the proposal of Russell *et al.* (8) that the A form is located on the inner membrane face. That the hypotonic swelling was effective in disrupting the outer membrane was shown by the complete inactivation of the adenylate kinase marker after less than 5 min of exposure to trypsin when mitochondria in 0.25 M sucrose were diluted 10:1 with water. Trypsin and chymotrypsin, at the high concentrations necessary to achieve appreciable rates of MAO inactivation, do appear to have some effect on outer membrane permeability under isotonic conditions, as indicated by the partial loss of adenylate kinase over the time period when MAO activity was monitored (Fig. 5). However, this does not detract from the essence of our argument as to the topography of the MAO multiple forms. The critical aspect of the data presented here is the difference between the effect of hypotonic disruption on the rate of protease inactivation of adenylate kinase and on that of the two forms of MAO. If some significant portion of one form of MAO were located on the inner face of the membrane, the large effect of hypotonic disruption on the susceptibility of adenylate kinase to protease inactivation would necessarily be reflected in a corresponding

change in the relative rates of inactivation of MAO-A and MAO-B.

Adenylate kinase appears, at most, to be only loosely associated with the mitochondrial membrane system and, as previously noted, can be released from disrupted mitochondria with EDTA (22). If all of the adenylate kinase were released into the medium by hypotonic shock alone, then its rapid destruction by trypsin and chymotrypsin could not be taken as an indication of access of the proteases to the intermembrane space. Since our studies have shown that a large portion of the adenylate kinase remains bound to the mitochondira following hypotonic disruption, whereas all of the activity is very rapidly inactivated by the proteases, good evidence is provided that these enzymes can enter the intermembrane space.

Considering the data for trypsin inactivation of MAO alone, the argument could be made that, although the catalytic site of one MAO form is oriented toward the interior of the mitochondrion, a particular peptide residue required for the maintenance of an active conformation and vulnerable to protease attack is exposed at the cytosolic face. That the β-chymotrypsin and *S. aureus* protease, with very different amino acid specificities, also showed no effect of hypotonic disruption on the relative rates of disappearance of MAO-A and MAO-B makes this proposition very unlikely. The increase in the rates of both MAO-A and MAO-B inactivation by chymotrypsin accompanying hypotonic disruption could be explained by the exposure of new peptide residues on both forms to cleavage as the outer membrane is disrupted.

The lack of an effect of hypotonic disruption on the relative rates of protease inactivation of MAO-A and MAO-B does not exclude the possibility of different orientations of the two forms on the same membrane surface. For example, the greater sensitivity of MAO-A to both trypsin and chymotrypsin might be explained by exposure of more of this peptide above the cytosolic membrane surface. This is not necessarily inconsistent with previous reports suggesting a greater dependence of MAO-A on bound lipid for stability (2, 3, 9, 23). A small number of specific lipid-protein interactions within the membrane may be critical to the maintenance of a catalytically active conformation while a significant portion of the peptide is, nevertheless, exposed above the membrane surface and thus vulnerable to attack by the proteases.

The reasons for the different findings in the present study and that of Russell *et al.* (8) with respect to distribution of the MAO-A and MAO-B activities between the two membrane faces is not clear, as both investigations utilized exactly the same methodology for disruption of the mitochondria and measurement of mitochondrial lysis. It is unlikely that the different tissues used could account for such dramatic differences in results. A possible alternative interpretation of the increased immunoinhibition of MAO-A which Russell *et al.* (8) observed on disruption of the mitochondria is a conformational change in the MAO following rupture of the outer membrane, which results in enhanced antibody binding and/or exposure of new antigenic sites. Since antisera for the studies by Russell *et al.* (8) were prepared to a solubilized, partially purified MAO, it is probable that antibodies would be present to antigenic sites which are masked in the enzyme's unperturbed membrane environment. Interpretation of their results is further complicated by certain methodological problems, including the following: (a) Only partial immunoinhibition was achieved with strongly differential effects between MAO substrates tested. (b) Activity was anomolously low for the A substrate, 5HT, making interpretations of changes questionable in the absence of statistical data. (c) Intact mitochondrial preparations showed a high degree of lysis (30–50%). (d) Their data showed considerable quantitative variation among individual tissue samples from four human autopsy subjects.

Recent studies have produced a variety of credible indirect evidence for the existence of the MAO-A and MAO-B forms as distinct peptides. These findings include separation of the two forms on sodium dodecyl sulfate acrylamide gels (24–26), identification of an active-site peptide fragment from *S. aureus* protease digestion unique to the B form (27), and preparation of a monoclonal antibody selective for MAO-B (28). The differences in the rates of inactivation of PEA oxidation by MAO in placenta and rat liver mitochondria in the present work add further credence to this notion. As pointed out under Results, the B substrate, PEA, is quite active with the MAO of both tissues; however, clorgyline and deprenyl inhibition patterns suggest that placenta contains only MAO-A. That MAO activity with PEA as a substrate is rapidly destroyed by trypsin in mitochondria from placenta but not from rat liver, whereas activity toward the A substrate, 5HT, is rapidly lost in both tissues, is consistent with the existence of two distinct MAO peptides in rat liver and a single species in placenta.

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