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Characterization and Quantitation of Monoamine Oxidases A and B in Mitochondria from Human Placenta

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SUMMARY

Monoamine oxidases (MAOs) A and B, flavin-containing enzymes found in the outer mitochondrial membrane, oxidize many important biogenic and xenobiotic amines. The two enzymes are expressed in many tissues, with some tissues containing primarily one form and others containing both. Although MAO in placental mitochondria is widely reported to be type A, some investigators have reported low levels of MAO B activity as well. Because placenta is considered the preferred source for purification of type A MAO, we have reinvestigated placental MAO by immunoblotting with monoclonal antibodies and active site labeling with the MAO-specific ligand [^{3}H]pargyline. We have confirmed that placental mitochondrial preparations contain MAO A and low but significant MAO B catalytic activity, as judged by accepted pharmacological criteria (deprenyl-sensitive β-phenylethylamine and benzylamine oxidation). Immunoblotting revealed polypeptides of sizes expected for both MAO A and B subunits in preparations of placental mitochondria, as well as in preparations of MAO A purified extensively from placenta by partitioning between dextran and polyethylene glycol polymers and chromatography on DEAE-Sepharose CL-6B. Both MAO A and B active sites could be quantitated in placenta by labeling mitochondrial preparations with the MAO-specific affinity ligand [^{3}H] pargyline, followed by immunoprecipitation with MAO A- and MAO B-specific monoclonal antibodies. These results indicate that MAO B activity and protein is consistently present in mitochondrial preparations of human placenta.

MAOs (EC 1.4.3.4) in the outer mitochondrial membrane (1) oxidize many biologically important amines, including the neurotransmitters serotonin, dopamine, and noradrenaline, and exogenous amines such as tyramine (2) and the potent dopaminergic neurotoxin MPTP (3). The two forms of MAO (A and B), originally defined by differences in inhibitor (4, 5) and substrate specificities (2), also differ in apparent molecular weight (6, 7), immunological properties (8–11), and amino acid sequence inferred from analysis of the human cDNAs (12).

MAO A and B are differentially expressed in a variety of tissues. Some, such as human liver and brain, contain both forms of MAO (4, 13), whereas others, such as human platelets (14) or placenta (15), contain primarily one form (MAO B and MAO A, respectively). In many tissues, the distribution and type of MAO varies dramatically among cell types, as for example in brain, where immunocytochemical studies suggest that the expression of the two enzymes in aminergic neurons is correlated with neurotransmitter phenotype (16, 17).

With the availability of monoclonal antibodies to MAO A and B (10, 11) and the recent cloning of their cDNAs (12, 18), it should be possible to investigate mechanisms that regulate expression of the respective genes and proteins in human cells and tissues. As background for such studies, there is a vast literature cataloging the distribution of MAO A and B catalytic activities in human cells and tissues. However, much of these data are of limited value in establishing the quantities of ratios of MAO A and B protein because the turnover numbers of MAO A and B for the substrates under the conditions of assay were not available or were not taken into account. In addition, because the traditional approach for discriminating MAO A and B activity requires the use of selective substrates and inhibitors, all of which show some activity with or inhibition of the alternative enzyme form, it has been difficult by these techniques alone to rule out expression of MAO A or B in cell or tissue preparations that contain high activities of the other MAO form.

Human placenta is an example of a tissue that was originally (15), and often thereafter, reported to contain MAO A but not MAO B. Because of the difficulty of separating the two enzymes, the apparent absence of MAO B in placental mitochondria has made these preparations the preferred starting source for purification of MAO A (15, 19). However, two reports have

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ABBREVIATIONS: MAO, monoamine oxidase; SDS, sodium dodecyl sulfate; Tween-20, polyoxyethylene sorbitan monolaurate; 5HT, 5-hydroxytryptamine, serotonin; PEA, β-phenylethylamine; TBS, Tris-buffered saline; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

suggested that low MAO B activity might be present as well. One report relied solely on catalytic activity and inhibitor data (20), the other on similar data plus reactivity with an MAO B-specific monoclonal antibody (10). Neither report provided any physical evidence that the MAO B-like activity was MAO B, nor was there any attempt to determine the amount of the enzyme or ratio of MAO A to MAO B protein.

In this report we confirm that, by accepted pharmacological criteria, all placental mitochondrial preparations examined contain low levels of MAO B activity in addition to the expected MAO A activity and that these preparations contain polypeptides with molecular weight, immunological properties, and pargyline-labeling properties expected for MAO A and MAO B.

Materials and Methods

Purified MAO A. Human MAO A, purified from placenta by two-phase polymer partitioning and chromatography on DEAE-Sepharose Cl-6B (19), was generously provided by Drs. James Salach and Walter Weyler (Department of Molecular Biology, Veterans Administration Medical Center, San Francisco, CA).

Monoclonal antibodies. With the exception of MAO A-4D3, the monoclonal antibodies to MAO A or B have been previously described [MAO A-7B10, MAO A-3C9, MAO A-7E10, MAO A-4F10 (11), and MAO B-1C2 (10)]. Hybridoma MAO A-4D3 was isolated basically as described previously (11), except that the immune spleen cells from female BALB/c BYJ mice (The Jackson Laboratory, Bar Harbor, ME), immunized with purified human MAO A (50 μg/injection, 250 μg of total MAO A/mouse), were fused to cells of the hypoxanthine-guanine phosphoribosyl transferase-deficient myeloma line P3/X63 Ag8.653 (21), which was obtained from Dr. Daniel Schulze (Department of Microbiology, University of Texas Medical Branch, Galveston, TX). Conditioned media were screened for antibodies that bound to microtiter plates coated with MAO A (enzyme-linked immunosorbant assay) or which immunoprecipitated catalytically active MAO A (11). One of 19 microcultures was positive for MAO A-specific antibody by both tests. Cells from this well (MAO A-4D3) were subcloned by limiting dilution, frozen, and stored in liquid nitrogen.

Preparation of ascites fluids. Ascites fluids containing antibodies from each hybridoma line were produced from pristane-primed female BALB/c BYJ mice that were treated intraperitoneally with 10^7 cells (22).

Preparation of extracts of placental mitochondria. Placentae were obtained from the Labor and Delivery Service of the Department of Obstetrics and Gynecology of the University of Texas Medical Branch (Galveston, TX), within 4 hr of delivery and were placed on ice. After removal of cord and membranes, the tissue was cut into small pieces, washed in ice-cold isotonic buffer (0.25 M sucrose, 0.5 mM EDTA, 10 mM K_3PO_4, pH 7.5), and homogenized using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY). Homogenates were centrifuged for 10 min at 2000 × g in a Beckman JA-10 centrifuge at 4°. The pellet was discarded and the supernatant was centrifuged for 15 min at 8000 × g. Pellets containing mitochondria were washed three times in isotonic buffer and stored at −80°.

Extracts were prepared by resuspending mitochondria in two volumes of 0.05 M potassium phosphate buffer (pH 7.3) and adding Triton X-100 to 1% (v/v). Suspensions were mixed for 20 min on a rotary shaker at room temperature. Insoluble material was removed by centrifugation at 10,000 × g. Protein concentration was determined (23) and extracts were stored at −80°.

Preparation of platelets. Samples of purified platelets were generously donated by Dr. Richard R. Fritz (Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX). Platelet preparations were isolated from outdated platelet-rich plasma as described for preparation of the external standard (24) and were stored at −80°.

Immunoprecipitation of MAO. Immunoprecipitation of catalytically active MAO A was performed as previously described (11), using goat anti-mouse IgG (heavy and light chain specific; Cooper Biomedical, Malvern, PA) and *Staphylococcus aureus* cells (Pansorbin, Calbiochem, San Diego, CA). Both supernatants and washed pellets were assayed for MAO A activity.

Immunoprecipitation of MAO B was performed as for MAO A, except that the antigen was [^{3}H]pargyline-labeled DEAE-enriched human platelet MAO B (2). Supernatants and washed pellets were assayed for [^{3}H]MAO B by mixing with liquid scintillation fluid (Hydrofluor; National Diagnostics, Somerville, NJ) and counted in an LKB 1217 liquid scintillation counter (LKB Instruments, Gaithersburg, MD; counting efficiency approximately 25%).

Assay of MAO activity. MAO activity was determined as described previously (10). The substrates were [^{14}C]serotonin (5HT; 50 Ci/mol, 100 μM final concentration), [^{14}C]PEA (50 Ci/mol, 10 μM final concentration), or [^{14}C]benzylamine (57 Ci/mol, 100 μM final concentration) in 0.1 M potassium phosphate buffer, pH 7.3 (all substrates from New England Nuclear, Boston, MA). Assay times were 10 min for 5HT and benzylamine and 30 min for PEA. Samples were diluted appropriately to assure linearity of the assays with time. Units of MAO activity are reported as nmol of substrate oxidized/hr/mg of protein.

Selective inhibition of MAO A or B. MAO A activity was inhibited by preincubation with clorgyline hydrochloride, and MAO B activity with L(−)-deprenyl hydrochloride (both inhibitors from Research Biochemicals, Natick, MA). Mitochondria were incubated for 20 min at 30° in 0.05 M potassium phosphate buffer (pH 7.3) containing 10^{-6} M inhibitor. (These inhibitor concentrations were selected after examining the selectivity of inhibition at 10-fold serial dilutions of inhibitor ranging from 10^{-9} to 10^{-3} M.) Mitochondria were washed in buffer minus inhibitor, and each sample was assayed in duplicate with all three substrates.

Polyacrylamide gel electrophoresis and transfer to nitrocellulose. For the gels in Figs. 1 and 2, samples were run on discontinuous SDS-polyacrylamide gels [8% acrylamide separating gel, 4% stacking gel, 14 × 10 × 0.15 cm slab; (26)] after dilution with sample buffer (final concentration: 2% SDS, 6% β-mercaptoethanol, 0.5 M Tris, pH 6.8) and heating for 2 min at 70°. Unless otherwise indicated, gels were run for 2 hr at 20 mA/gel, followed by 2 hr at 40 mA/gel. The gel in Fig. 3 was polymerized with riboflavin (0.0005%) and pre-run for 3 hr at 30 mA before sample application, in an attempt to minimize the formation of artifactual, faster running bands in the purified MAO A preparation (19).

Proteins were transferred to nitrocellulose in 0.025 M Tris, 0.192 M glycine, 20% methanol (27), for 17 hr at 75 mA at 23°. These conditions permitted efficient transfer of MAO, even though some other proteins remained in the gel. After transfer, some sections of nitrocellulose were stained for total protein in Amido black 10B (Bio-Rad, Richmond, CA) (0.1% dissolved in 7% acetic acid), whereas others were incubated with antibody detectors for specific staining of MAO A or B.

Binding of antibodies to nitrocellulose. After electrophoretic transfer, nitrocellulose filters were blocked with 5% Carnation nonfat dried milk in TBS (0.17 M NaCl, 0.01 M Tris, pH 7.5) for 30 min, with several changes of buffer, and then incubated overnight at 23° in blocking buffer that contained primary antibody and 0.1% (v/v) Tween-20. The addition of Tween-20 at this step was crucial for sensitive detection of MAO A and MAO B.

Bound antibodies were detected using a Vector Laboratories ABC (Figs. 1 and 2) or Elite kit (Fig. 3; Burlingame, CA). The Elite kit is claimed to be 5 times more sensitive for antibody detection than the standard ABC kit. After washing with TBS, filters were incubated for 1 hr in TBS containing biotinylated anti-mouse IgG, washed, and then incubated for 1 hr in avidin plus biotinylated horseradish peroxidase. Avidin and biotinylated horseradish peroxidase, mixed according to manufacturer's directions, were diluted 1:100 before use in order to reduce avidin binding to proteins other than biotinylated antibody in crude extracts. The peroxidase substrate used for color development

was 4-chloro-1-naphthol (Bio-Rad) (60 mg dissolved in 20 ml of ice-cold methanol and added to 100 ml of TBS containing 60 μl of 30% H_2O_2, immediately before use).

Comigration of [^{3}H]pargyline-labeled MAO B and band immunostained with MAO B-1C2. To verify that the band from platelets that stained with MAO B-1C2 in immunoblots was MAO B, 59 μg of [^{3}H]pargyline-labeled DEAE-enriched platelet MAO B [containing 160,000 dpm in 590 ng of MAO B (25)] were electrophoresed and transferred to nitrocellulose as described above. After immunological staining with MAO B-1C2 ascites fluid, the corresponding lane was cut into 28 0.5-cm slices and counted in a scintillation counter.

Quantitation of MAO A and B active sites using [^{3}H]pargyline. Mitochondria from a representative placenta were incubated (200 μl containing 2.98 mg) for 3 hr at 30° in 0.05 M potassium phosphate buffer (pH 7.3) containing 5×10^{-6} M [^{3}H]pargyline hydrochloride (22.5 Ci/mmol; New England Nuclear) that had been dried under nitrogen immediately before use. Control samples of mitochondria, containing only buffer, were incubated in parallel. After incubation, mitochondria were washed six times in buffer minus inhibitor. Both control and labeled mitochondria were assayed with 100 μM 5HT for determination of per cent inhibition by pargyline.

The quantity of pargyline bound to MAO A and B was determined by immunoprecipitation, as previously described (11). The labeled mitochondria were extracted with 1% Triton X-100 and the proportion of extractable counts as a function of original mitochondrial protein was determined. Aliquots (20,000 dpm) were mixed with a 1/100 dilution of MAO A-4D3 or MAO B-B1C2 ascites fluid. This antibody dilution gave maximal immunoprecipitation of the enzymes (>90%) under these conditions. Total immunoprecipitable label (representing total [^{3}H]pargyline-labeled MAO) was determined by immunoprecipitation using a mixture of both ascites fluids, 1/100 dilutions of each. Loss of counts from immunoprecipitation supernatants was determined by liquid scintillation counting and nmol of MAO/mg of mitochondrial protein were calculated. Stoichiometries of 1 FAD/MAO subunit (28) and 1 pargyline/FAD were assumed. The concentration of MAO A or B, in nmol/mg of mitochondrial protein, was equal to $N/(F_1 \times F_2 \times 2)$, where N = nmol of pargyline immunoprecipitated/mg of mitochondrial protein, F_1 = the measured fraction of label extracted with Triton X-100, F_2 = the measured fraction of MAO inactivated by pargyline (for MAO A) or 1 (for MAO B), and 2 = number of FAD/MAO molecule.

Results

Detection of MAO B activity in placental mitochondria. Mitochondrial preparations from four placentae were assayed for oxidation of 5HT, PEA, and benzylamine after preincubation with varying concentrations of MAO A- and MAO B-specific inhibitors (clorgyline and deprenyl, respectively). In each preparation, clorgyline (10^{-6} M) inhibited 5HT oxidation by placental mitochondria by 99%, suggesting that this concentration of clorgyline inhibited MAO A almost completely under our conditions. In the identical, clorgyline-treated preparations, PEA oxidation was inhibited by 90% and benzylamine oxidation by 56% (in three placentae) or 10% (one placenta). These data are summarized in Table 1.

To determine whether any of the PEA- and benzylamine-oxidizing activities not inhibited by 10^{-6} M clorgyline were attributable to MAO B, the residual activities were assayed after further treatment with 10^{-6} M deprenyl. More than half of the PEA- and benzylamine-oxidizing activities remaining after clorgyline treatment were inhibited by 10^{-6} M deprenyl. The mean and range of PEA- and benzylamine-oxidizing activities resistant to clorgyline but sensitive to deprenyl are shown in Table 1, columns 2 and 3. This relatively clorgyline-resistant, deprenyl-sensitive, PEA- and benzylamine-oxidizing activity

TABLE 1

MAO activity in placental mitochondria

Placental mitochondria (20 μl; 60–80 μg) were inhibited with 10^{-6} M clorgyline alone or 10^{-6} M clorgyline plus 10^{-6} M deprenyl, as described in Materials and Methods. Control samples were incubated in buffer alone and diluted 1/10 before assay with PEA or 5HT. Values are average ± standard deviation of four placentae assayed in duplicate, and ranges of values are in parentheses.

Activity component	Oxidizing Activity with Indicated Substrates		
	Serotonin	PEA	Benzylamine
	nmol/hr/mg		
Total	803 ± 183 (439–979)	44.5 ± 14.5 (17–78)	29.4 ± 10.6 (13.6–49)
Clorgyline-sensitive	768 ± 166 (430–944)	36.6 ± 16.2 (10–69)	10.9 ± 4.6 (5–17)
Deprenyl-sensitive	3.6 ± 2.4[a] (1.2–6.0)	4.7 ± 1.6 (1.3–6)	7.1 ± 2.3[b] (3.7–9)

[a] Average of two placentae; undetectable in others.
[b] Average of three placentae, see Results.

TABLE 2

Immunoprecipitation of human MAO A but not MAO B by MAO A-4D3

Antibody Source	Immunoprecipitation of MAO A activity[a]		Immunoprecipitation of [^{3}H]MAO B (Amount in Pellet)[b]
	Activity recovered	Amount in Pellet	
	nmol/hr	%	*% dpm*
MAO A-4D3	1.42 ± 0.2	97	3 ± 2
MAO A-7B10	1.62 ± 0.3	95	1 ± 0.4
MAO B-1C2	1.78 ± 0.04	2	86 ± 1

[a] Samples of Triton X-100 extract of placenta mitochondria (20 μg of protein) were immunoprecipitated with MAO A-4D3, MAO A-7B10, and MAO B-1C2, as described in Materials and Methods. Values are averages and standard deviations of triplicate assays.
[b] Samples of [^{3}H]pargyline-labeled, DEAE-enriched platelet MAO B containing 2 μg of protein (5500 dpm in 20 ng of MAO B) were immunoprecipitated with MAO A-4D3, MAO A-7B10, and MAO B-1C2, as described in Materials and Methods. Values are averages and standard deviations of triplicate assays.

fulfills the accepted pharmacological criteria for MAO B. Defined in this way, the amount of MAO B activity appeared to be considerably higher in one placenta (34 nmol/hr/mg of protein clorgyline-resistant, deprenyl-sensitive, benzylamine-oxidizing activity) than in the other three (7.1 ± 2.3 nmol/hr/mg; Table 1).

Characterization of immunoblotting assay for MAO A and B. Because no monoclonal antibody-mediated immunoblotting assay for MAO A or MAO B has been described, we examined the parameters for satisfactory immunoblotting of MAO A and B. MAO A-4D3 ascites fluids were consistently superior to previously described MAO A-specific monoclonal antibodies in immunoblotting of MAO A. Preliminary experiments (without the nonionic detergent Tween-20) showed that high concentrations (1/30, v/v) of ascites fluid from hybridoma lines MAO A-7B10, -7E10, -4F10, and -3C9 did not stain 25 μg of purified human MAO A after electrophoresis in SDS-polyacrylamide gels and transfer to nitrocellulose. However, staining of MAO A polypeptide by MAO A-4D3 antibody was observed under the same conditions (data not shown). Despite the superiority of MAO A-4D3 in immunoblotting, this antibody is similar the other MAO A-specific antibodies in that it was elicited to native, catalytically active, human MAO A, immunoprecipitated the catalytically active enzyme (Table 2), and bound to MAO A-coated microtiter plates (enzyme-linked immunosorbent assay; data not shown). MAO A-4D3 antibody does not bind MAO B, because it immunoprecipitated little or

no [^{3}H]pargyline-labeled human platelet MAO B (Table 2) or MAO B catalytic activity (data not shown).

Without addition of Tween-20 to the primary incubation buffer, the immunoblotting sensitivity for MAO A, even with MAO A-4D3 ascites fluid, was insufficient to stain the enzyme in 500 μg of Triton X-100 extract of placental mitochondria (estimated to contain about 500 ng of MAO A; data not shown) or 500 ng of purified enzyme (Fig. 1A, *lane 2*). However, addition of Tween-20 (0.1%, v/v) to the primary antibody incubation buffer resulted in clear staining of 500 ng of purified enzyme [Fig. 1A, *lane 4* (0.1% Tween-20)] and a single band of identical molecular weight in 35–140 μg of Triton X-100-extracted, placental mitochondrial protein (Fig. 1A, *lane 3*; Fig. 1B, *lanes 1* and *2*). Neither omission of β-mercaptoethanol from the SDS-sample buffer (Fig. 1B, *lane 3*) nor denaturation of samples at room temperature rather than 70° resulted in any further gain in sensitivity (data not shown).

Comparison of staining of purified MAO A by Amido black or MAO A-4D3 antibodies showed that immunoblotting stained the predominant protein band in the preparation [compare Fig. 1B, *lanes 4* and *6*; *lane 4*, inadvertently cut in half, was stained both by Amido black (*right*) and MAO A-4D3 (*left*)]. Minor protein bands that migrated faster than the predominant protein in the purified MAO A preparation were stained by both Amido black and antibody. These bands probably correspond to MAO A molecules migrating anomalously during electrophoresis, as described by Weyler and Salach (19), or to partial proteolytic breakdown products. Unreduced MAO A ran as a more diffuse band than reduced MAO A (Fig. 1B, *lanes 3* and *5*).

The conditions optimal for MAO A staining by MAO A-4D3 (plus Tween 20) proved to be satisfactory for MAO B-1C2 staining of MAO B as well. MAO B-1C2 ascites fluid stained a single band of 59,000 molecular weight in immunoblots of whole platelets (Fig. 2A, *lane 3*). When [^{3}H]pargyline-labeled, DEAE-enriched, platelet MAO B was electrophoresed (Fig. 2A, *lane 2*), the 59,000 molecular weight band stained by MAO B-1C2 ascites fluid coincided with a peak of ^{3}H that contained 70% of the counts recovered from the nitrocellulose strip (data not shown; no other individual half centimeter section contained more than 6% of the total cpm). Omission of Tween-20 from the monoclonal antibody incubation buffer markedly diminished the staining of MAO B by antibodies from MAO B-1C2 (compare Fig. 2B, *lanes 4* and *7*).

Analysis of platelet and placental extracts for MAO A and B protein. Antibody produced by MAO B-1C2 but not MAO A-4D3 stained a single band of approximately 59,000 molecular weight (compare Fig. 3, *lanes 3* and *7*) in a nitrocellulose transfer of 230 μg of platelet protein. No band corresponding in molecular weight to MAO A was stained in platelets by either antibody, as expected.

MAO A-4D3 and MAO B-1C2 stained different bands of approximately 66,000 and 59,000 apparent molecular weight, respectively, in nitrocellulose transfers of Triton X-100 extracts of placental mitochondria (Fig. 3, *lanes 4* and *6*). That the bands stained by the two antibodies were different was confirmed by the appearance of both bands in placental extracts stained with a mixture of both antibodies (Fig. 3, *lane 5*). The band stained by MAO B-1C2 ascites fluid in placental extracts comigrated with the band in total platelet protein stained by the same antibody and is probably MAO B. A faint band of identical molecular weight was also stained by MAO B-1C2 in a preparation of purified human MAO A that was isolated from placenta and was provided to us by Drs. Weyler and Salach (19) (Fig. 3, *lane 8*; more clearly demonstrated in Fig. 2B, *lane 5*).

Quantitation of MAO A and B active site concentration using [^{3}H]pargyline labeling. Mitochondria from a placenta

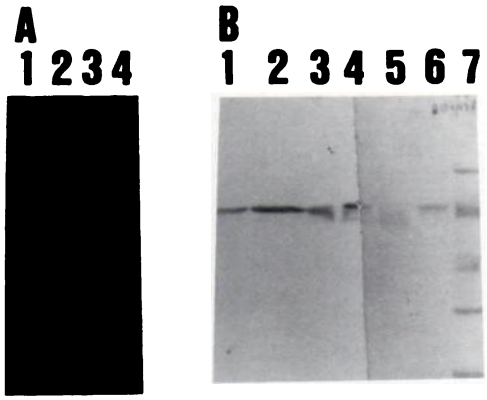

Fig. 1. Staining of human MAO A by MAO A-4D3 antibodies. Nitrocellulose transfers of 8% SDS-polyacrylamide gels were stained as described in Materials and Methods. A, Effect of Tween-20 on staining. Primary antibody incubation with 1/30 MAO A-4D3 ascites fluid in buffer lacking (*lanes 1* and *2*) or containing (*lanes 3* and *4*) 0.1% Tween-20. *Lanes 1* and *3* contain 35 μg of placental mitochondrial extract; *lanes 2* and *4* contain 0.5 μg of purified human MAO A. B, MAO A-4D3 antibodies stain MAO A. *Lanes 1–4* were stained with 1/30 MAO A-4D3 ascites fluid. *Lanes 5–7* were stained with 0.1% Amido black. *Lane 1*, 35 μg of placental mitochondrial extract; *lane 2*, 140 μg of placental mitochondrial extract; *lanes 3* and *5*, 0.5 μg of purified human MAO A treated at 70° in sample buffer without β-mercaptoethanol; *lanes 4* and *6*, 0.5 μg of purified human MAO A; *lane 7*, Bio-Rad low molecular weight standards. Molecular weights are (from *top* to *bottom*) phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,300.

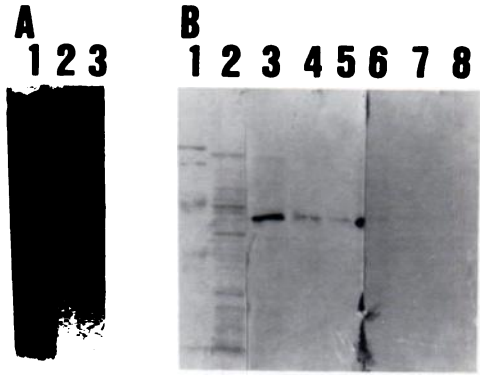

Fig. 2. Staining of human MAO B by MAO B-1C2 antibodies. Nitrocellulose transfers of 8% SDS-polyacrylamide gels were stained as described in Materials and Methods. A, MAO B-1C2 antibodies stain MAO B. *Lane 1* is stained by 0.1% Amido black; *lanes 2* and *3* are stained by 1/200 MAO B-1C2 ascites fluid. *Lanes 1* and *3*, 230 μg of platelet protein; *lane 2*, 59 μg of [^{3}H]pargyline-labeled DEAE-enriched platelet MAO B. B, Effect of Tween-20 on staining of MAO B by MAO B-1C2 antibodies. *Lanes 1* and *2* were stained by 0.1% Amido black; *lanes 3–8* were stained with 1/30 MAO B-1C2 containing (*lanes 3–5*) or lacking (*lanes 6–8*) 0.1% Tween-20. *Lane 1*, Bio-Rad high molecular weight markers, 2 μg of each protein. Molecular weights are (from *top* to *bottom*) myosin, 250,000; β-galactosidase, 140,000; phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 43,000. *Lanes 2, 3,* and *6*, 35 μg of placental mitochondrial extract; *lanes 4* and *7*, 2 μg of [^{3}H]pargyline-labeled DEAE-enriched platelet MAO B; *lanes 5* and *8*, 0.5 μg of purified human MAO A.

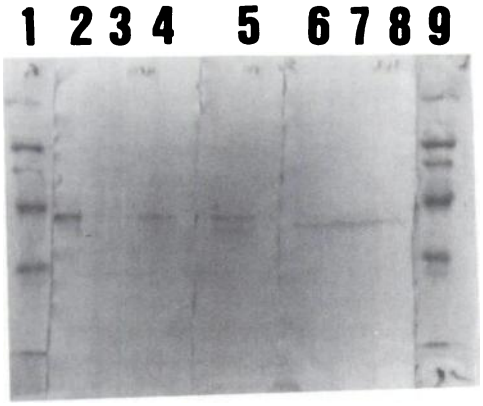

Fig. 3. Staining of MAO A and MAO B in human placenta. A nitrocellulose transfer of an 8% SDS-polyacrylamide gel was stained as described in Materials and Methods. *Lanes 1* and *9* were stained with 0.1% Amido black (high molecular weight markers as in Fig. 2); *lanes 2–4* were stained with 1/200 MAO A-4D3 ascites fluid (*lane 2*, 1 μg of purified MAO A; *lane 3*, 230 μg of platelet protein; *lane 4*, 100 μg of Triton X-100 extract of placental mitochondria). *Lane 5* was stained with a mixture of 1/200 MAO A-4D3 and 1/1000 MAO B-1C2 ascites fluids (100 μg of Triton X-100 extract of placental mitochondria); *lanes 6–8* were stained with 1/1000 MAO B-1C2 (*lane 6*, 100 μg of Triton X-100 extract of placental mitochondria; *lane 7*, 230 μg of platelets; 4 μg of purified human MAO A).

were incubated with 5×10^{-6} M [^{3}H]pargyline. Pargyline inhibits MAO by covalently binding to the active site flavin in a stochiometric manner (29). Although pargyline shows slight preference for inhibiting MAO B, under our conditions 95% of MAO A activity, as measured by 5HT oxidation, was inhibited as well, suggesting that both MAO A and B active sites were ≥95% saturated. After labeling, the mitochondria were extracted with 1% Triton X-100, aliquots were immunoprecipitated with 1/100 MAO A-4D3 or MAO B-1C2 ascites fluid, and the concentration of MAO A and B active sites was calculated as described in Materials and Methods. Results indicated that placental mitochondrial extract had a 22.5:1 ratio of MAO A to B active sites (76 pmol of MAO A/mg of protein, compared with 3.4 pmol of MAO B/mg protein; total MAO A and B protein from 261 g of tissue, 44.3 nmol of MAO A and 1.97 nmol of MAO B). This compares with a 24:1 MAO A to B ratio of PEA-oxidizing activity (34 nmol/hr/mg were sensitive to 10^{-6} clorgyline and 1.4 nmol/hr/mg were resistant to 10^{-6} M clorgyline and sensitive to 10^{-6} M deprenyl). A second placenta, labeled with pargyline solely to determine the ratio of MAO A to B active sites, had a ratio of 7:1. Although both placentae had similar specific activity of serotonin oxidation, the second had higher than average MAO B activity (deprenyl-sensitive benzylamine-oxidizing activity, 34 nmol/hr/mg), suggesting that it had a higher concentration of MAO B active sites.

Discussion

Although human placenta is often reported to contain type A but not type B MAO, we have confirmed two previous reports (10, 20) that placental mitochondria contain low MAO B activity, defined by accepted pharmacological criteria (PEA- or benzylamine-oxidizing activity inhibitable by 10^{-6} M deprenyl and relatively resistant to 10^{-6} M clorgyline; Table 1). That the activity detected is MAO B is supported by the following evidience: 1) detection of activity with appropriate substrate and inhibitor specificity, 2) immunological staining of polypeptides of the molecular weights expected for MAO B subunits, 3) binding of [^{3}H]pargyline, and 4) specific immunoprecipitation of [^{3}H]pargyline-labeled material with an MAO B-specific monoclonal antibody.

The ease with which we detected MAO B polypeptide in placental mitochondria by immunoblotting was surprising, considering the very low MAO B catalytic activity present. In fact, under optimal conditions, the intensities of staining of MAO A and B in equal quantities of placental mitochondrial protein were comparable, despite the high ratio of MAO A to MAO B activity. Active site labeling with [^{3}H]pargyline, followed by the separation of MAO A from MAO B by immunoprecipitation with type-specific monoclonal antibodies, also revealed a high ratio of MAO A to B active sites (76 ± 4.9 pmol of MAO A and 3.4 ± 0.41 pmol of MAO B per mg, for an A to B ratio of 22.5:1). The MAO B active site concentration in placental mitochondria, although low, is greater than the reported concentration of MAO A active sites in human liver mitochondria determined by [^{3}H]pargyline binding (30).

We also attempted to estimate the quantities of MAO A and B protein by image analysis of immunoblots containing unknowns run adjacent to graded quantities of purified enzyme. The results suggested that the sensitivity of detection of MAO A protein in immunoblots was only about one tenth that of MAO B. Therefore, despite comparable staining intensities of MAO A and B by their respective monoclonal antibodies, there was considerably less MAO B than MAO A protein in the placental preparations (108 ± 54 pmol of MAO A and 3.2 ± 2 pmol of MAO B per mg; data not shown). These figures for MAO A and B protein concentration agree well with values obtained from [^{3}H]pargyline labeling. However, in our hands, the quantitation of protein by staining intensity in immunoblots had a large coefficient of variation. Consequently, more work would be required before we would recommend immunoblotting as a reliable method for quantitating MAO A or B protein.

Although 10 μM PEA is oxidized primarily by MAO B in liver and brain (31), we have observed that the majority of PEA oxidation in placental mitochondria is sensitive to clorgyline (MAO A). This suggests that PEA, even at 10 μM, is not highly selective for MAO B, and it is only the relatively high ratio of MAO A to B active sites in placenta, compared with liver or brain, that leads to the predominant oxidation of PEA by MAO A in this tissue. In the first placenta in which the concentrations of MAO A and B active sites were determined, there was an A to B ratio of 22.5:1 and a ratio of A to B PEA oxidation of 24:1, suggesting that PEA was actually oxidized slightly better by MAO A than MAO B at this concentration. However, PEA oxidation by MAO B in this placental preparation could only be estimated after prior inhibition of MAO A with 10^{-6} M clorgyline, which may have also inhibited some of the MAO B. It is possible, therefore, that in an uninhibited placental preparation PEA at 10 μM is oxidized more rapidly per molecule of enzyme by MAO B than by MAO A. Nevertheless, the relative insensitivity of 10 μM PEA oxidation to deprenyl in uninhibited preparations indicates that, even at this concentration, PEA should not be considered a highly selective MAO B substrate.

Various protocols have been described for measuring MAO A and B active site concentrations, based upon stoichiometric binding of suicide inhibitors (32–34). Pargyline, currently the only commercially available radiolabeled compound of this

type, binds to both MAO A and B if used at sufficient concentrations (13). Measurement of labeled pargyline binding to mitochondria can be used to estimate active site concentrations of MAO A and MAO B, provided the enzymes can be distinguished from each other and from possible nonspecifically bound pargyline. Parkinson and Callingham (33) and Kalaria *et al.* (30) quantitated MAO A and B by inhibiting one form with more specific inhibitors (such as clorgyline or deprenyl), saturating the residual active MAO with labeled pargyline, and quantitating the bound label. However, because most MAO inhibitors exhibit some reactivity for the alternate MAO form, the conditions required to inhibit one MAO form without significantly inhibiting the other must be carefully defined and can vary from tissue to tissue and preparation to preparation.

In contrast, we used highly specific monoclonal antibodies rather than inhibitors to discriminate between [^{3}H]pargyline-labeled MAO A and B. There are three advantages to the immunological approach. First, mitochondria are labeled with [^{3}H]pargyline without prior incubation with inhibitor. This avoids the possible underestimation of the quantity of the MAO form of interest that might result from cross-inhibition with selective inhibitors. Second, it appears that monoclonal antibodies discriminate MAO A and B more efficiently than do inhibitors. Inhibitor specificity is concentration dependent but extensive studies of monoclonal antibodies generated against MAOs have revealed no cross-reaction between them and the alternate enzyme form (10, 11, 35). Third, the immunoprecipitation step permits monitoring of, and if necessary correction for nonspecific binding of pargyline to mitochondria, because only pargyline that immunoprecipitates with the appropriate monoclonal antibody is assumed to represent MAO-bound label.

The cellular source of the MAO B detected in placental tissue is unknown. Immunocytochemical staining of placental tissue slices with monoclonal antibodies revealed marked immunofluorescence for MAO A in synctiotrophoblasts, but only faint diffuse staining for MAO B (36). Failure to detect marked immunocytochemical staining for MAO B in placenta is not necessarily inconsistent with the MAO A:MAO B ratios reported here, provided MAO B is not highly concentrated in a subpopulation of cells. We estimate that MAO B protein constituted no more than 5–14% of the total MAO concentration (MAO A plus MAO B).

Because placenta is rich in blood and platelets are a well recognized source of MAO B (14), it is important to consider whether the MAO B activity and protein detected here might derive from blood. We recovered 1.97 nmol of MAO B from the placenta, which contained a 22.5:1 ratio of MAO A to B active sites. From the MAO B contained in the platelet preparation used here, we estimate that the blood from which it was derived contained no more than 0.25 pmol of MAO B/ml.[1] Therefore, the MAO B recovered in the placenta would correspond to the MAO B in 1970/0.25 or 7900 ml of blood, an unlikely amount, unless virtually all of the platelets in maternal and fetal blood were sequestered in the placenta. Lymphocytes may contain MAO B (37) but would be expected to contribute less MAO B than platelets. We were unsuccessful in attempts to remove the MAO B protein by repeated washing of diced fragments of placental tissue. Although the hemoglobin concentration in the resulting homogenates could be decreased routinely by 70–75%, compared with unwashed tissue, the relative staining intensity of MAO A to B in the mitochondrial preparations from these washed and unwashed fragments was indistinguishable after immunoblotting with a mixture of MAO A-4D3 and MAO B-1C2 (data not shown).

Lewinsohn and Sandler (20), who detected deprenyl-sensitive benzylamine-oxidizing activity in placenta, speculated that placental MAO B might be present in the vascular endothelium of the placenta. This would be consistent with the recent observation that MAO B activity is present in the vascular tissue of the cerebrum, where it may play a role in the blood-brain barrier (30).

Further support for the possibility that MAO B might be synthesized by cells intrinsic to the placenta comes from preliminary molecular biological studies. Bach *et al.* (12) reported low levels of MAO B mRNA in RNA isolated from this tissue. Furthermore, we have isolated, from a human placental cDNA library, a recombinant λ bacteriophage that hybridizes strongly and specifically to two oligonucleotides (a 59-mer and a 48-mer)[2] that encode segments of MAO B cDNA sequence published by Bach *et al.* (12). The phage does not hybridize under the same conditions to a third oligonucleotide, which encodes a corresponding region of nucleotide sequence from human MAO A. Preliminary restriction enzyme analysis of this phage suggests that it contains at least the 3′-terminal half of MAO B cDNA, including the FAD-binding region. If confirmed, these molecular biological data would argue strongly for a cellular source of placental MAO B other than platelets, because platelets synthesize no significant protein and contain little or no mRNA.

Regardless of the cellular source of MAO B, it is important to recognize that placental mitochondria, as commonly prepared, as well as preparations of placental MAO A purified by an established protocol (19) contain small amounts of MAO B protein. If the presence of placental MAO B in placental preparations were ignored, the MAO A activity towards substrates highly selectively oxidized by MAO B (such as benzylamine) might be considerably overestimated.

Tween-20 (0.1%, v/v) is commonly used to decrease nonspecific binding in immunoblots (reviewed in Ref. 38). When it was added to the primary antibody incubation solution, the detergent greatly increased the sensitivity of detection of MAO A and B polypeptides in immunoblots. Previous investigators noted that Tween-20 enhanced the binding of calmodulin to nitrocellulose-bound proteins (39) and speculated that the detergent increased binding of ligand by partially removing the protein from the nitrocellulose, thereby freeing the nitrocellulose-bound polypeptides to acquire a more native conformation. Our immunoblotting results suggest that the addition of Tween-20 to the primary antibody incubation step might be a simple

[1] The amount of MAO B protein in whole blood was calculated from the amount of MAO B in the platelet preparation from pooled outdated platelets that was used here and that was prepared and characterized for MAO B content as described in Fritz *et al.* (24). Each milliliter of this preparation, containing platelets from 45 ml of platelet-rich plasma, contained 2.69 μg of MAO B (0.54 μg of MAO B/mg of protein), as determined by [^{3}H]pargyline binding. Because this MAO B was derived from 45 ml of platelet-rich plasma, the concentration of MAO B in the unconcentrated preparation was 0.06 μg of MAO B/ml, or 0.25 pmol of MAO B/ml (0.060 μg of MAO B/ml of blood)/[(120 ng/pmol B) × 2] = 0.25 pmol of MAO B/ml), assuming a molecular weight of 120,000 for MAO B and one FAD/subunit. The concentration of MAO B in whole blood would be slightly lower than that in platelet-rich plasma, because of the volume occupied by blood cells.

[2] R. M. Denney, E. L. Parker, and L. A. Riley, unpublished observations.

way to enhance the sensitivity of other immunoblotting systems.

In conclusion, we have confirmed by accepted pharmacological criteria that both MAO A and B activities are present in placental mitochondrial preparations. We have demonstrated further by immunoblotting that the molecular weight of the placental MAO B is indistinguishable from that of the well characterized platelet enzyme and we have used a simple combination of active site labeling and immunoprecipitation to measure the ratio and quantity of MAO A and B protein in placental preparations. The techniques developed here to detect and quantitate MAO A and B protein in placental mitochondria constitute improved methods, which should lead to more accurate values for concentrations of MAO A and B protein in many human tissues.

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