Immunological Uniqueness of Human Monoamine Oxidases A and B: New Evidence from Studies with Monoclonal Antibodies to Human Monoamine Oxidase A¹

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Abstract

Monoamine oxidase (EC 1.4.3.4; MAO) is the primary enzyme responsible for the intraneuronal degradation of biogenic amines in the central nervous system. An understanding of the physiological significance of the functional and regulatory differences between the two forms of the enzyme, MAOs A and B, would be facilitated by the availability of antibodies specific for the two forms of the enzyme. We previously isolated and characterized a monoclonal antibody (MAO B-1C2, previously designated MAO-1C2) which binds human MAO B but not A. We describe here four new monoclonal antibodies (designated MAO A-3C9, A-4F10, A-7B10, and A-7E10) which were elicited to highly purified MAO A from human placenta and which, in the presence of anti-mouse IgG and *Staphylococcus aureus*, immunoprecipitate >90% of the catalytically active purified MAO A. MAO A-3C9 appears to have a lower affinity for purified MAO A than the other three antibodies and does not immunoprecipitate either MAO A or MAO B from human platelets or from Triton X-100 extracts of human placental and liver mitochondria. MAO A-4F10, A-7B10, and A-7E10 immunoprecipitate catalytically active MAO A from Triton X-100 extracts of human placental and liver mitochondria, but not catalytically active MAO B from either platelets or from Triton X-100 extracts of human liver mitochondria. Collectively, these anti-MAO monoclonal antibodies reveal unique epitopes on human MAO A not shared by MAO B, and at least one epitope on MAO B not shared by MAO A. These immunochemical differences support the hypothesis that MAO A and MAO B are different proteins, presumably isozymes.

Monoamine oxidase (MAO; EC 1.4.3.4) is an integral protein of the outer mitochondrial membrane (Greenawalt and Schnaitman, 1970) which oxidizes biogenic and xenobiotic amines. MAOs A and B rapidly oxidize 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

(MPTP),³ a neurotoxin which selectively destroys nigrostriatal dopaminergic neurons and produces Parkinsonian-like symptoms in humans and experimental animals (Chiba et al., 1984; Gessner et al., 1984; Langston et al., 1984; Markey et al., 1984; Salach et al., 1984). Both forms of MAO are irreversibly inactivated *in vitro* by MPTP or its oxidation products (Singer et al., 1985). MAO is present in virtually all mammalian tissues and has received considerable attention in the central nervous system because of its potential role in the etiology of psychiatric disease (Wyatt et al., 1980; Sandler et al., 1981; Fowler and Ross, 1984; for a contrasting view, see Fowler et al., 1982).

Differences in the substrate and inhibitor preferences of MAO from a variety of tissues indicates that there are two functional types of MAO: MAO A, which preferentially oxidizes low concentrations of 5-hydroxytryptamine (5-HT) and is inhibited by low concentrations of clorgyline (Johnston, 1968), and MAO B, which preferentially oxidizes low concentrations of β -phenylethylamine (PEA) and is inhibited by low concentrations of deprenyl (Knoll and Magyar, 1972). Many tissues, including human liver (Murphy and Donnelly, 1974), express both MAO A and MAO B activity, whereas a few tissues, including human placenta (Egashira, 1976) and human platelets (Donnelly and Murphy, 1977), express only one form (MAO A or B, respectively). MAOs A and B also differ in the extent to which they are regulated by steroid hormones (Edelstein and Breakefield, 1981; Mazumder et al., 1980).

MAOs A and B are similar but probably not identical in polypeptide structure. Both enzymes are dimers which contain an essential, covalently bound flavin adenine dinucleotide (FAD) cofactor at a molar ratio of 1 FAD/2 subunits of enzyme (Kearney et al., 1971; Minamiura and Yasunobu, 1978; Salach, 1979; Salach and Detmer, 1979). The active site pentapeptide containing the flavin residue is identical in MAO A and MAO B from tissues of several species (Nagy and Salach, 1981; Yu, 1981). Subunits of MAO B have equal mobility in SDS-polyacrylamide gel electrophoresis; the same is true for MAO A. However, for all species examined to date, MAO B subunits have a slightly higher mobility on these gels than do MAO A subunits (apparent M_r = ~60,000 to 64,000 for MAO B; 64,000 to 67,000 for MAO A) (Callingham and Parkinson, 1979; Brown et al., 1980; Cawthon and Breakefield, 1979, 1983; Cawthon et al., 1981). When impure preparations of MAOs A and B labeled with the irreversible inhibitor ³H-pargyline were subjected to partial proteolytic

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³ The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; FAD, flavin adenine dinucleotide; 5-HT, 5-hydroxytryptamine (serotonin); MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate-buffered saline; PEA, phenylethylamine.

digestion, the FAD-containing (pargyline-bound) subunits of the enzymes appeared to be similar, but not identical (Cawthon and Breakefield, 1979).

The molecular basis for the observed functional, structural, and regulatory differences between MAO A and MAO B is unknown. MAO A and MAO B may be distinct molecular species, coded and regulated by distinct genes. However, it is possible that MAO A and B activities arise from a single gene and differ in function because of post-transcriptional or post-translational events or non-covalent association of MAO peptides with constituents of the mitochondrial membrane (Houslay and Tipton, 1973; Huang and Faulkner, 1980).

If MAOs A or B are different proteins, they should be immunologically distinguishable. Comparative studies of MAO A and MAO B with polyclonal antisera have yielded conflicting results. Whereas some investigators have prepared antisera that exhibit considerable MAO A or B specificity (McCauley and Racker, 1973; Powell and Craig, 1977; Craig et al., 1982; Pintar et al., 1983), others have reported that MAOs A and B are immunologically indistinguishable (Dennick and Mayer, 1977; Russell et al., 1979). Monoclonal antibodies (Köhler and Milstein, 1976) offer theoretical advantages in comparative studies of MAOs A and B, because they recognize single epitopes and can be produced in unlimited quantities. Until now, there has been only one monoclonal antibody to MAO, an antibody which was elicited to and recognizes human MAO B but not human MAO A (Denney et al., 1982a, b, 1983). This antibody was previously designated MAO-1C2, but we will in the future refer to it as MAO B-1C2 to clarify its specificity for MAO B. The specificity of MAO B-1C2 for MAO B demonstrates that at least one discrete epitope on human MAO B is not found on MAO A. However, evidence obtained with only one antibody cannot address the extent of immunological differences between the two enzymes. Monoclonal antibodies to MAO A were needed to compare other epitopes on the two enzymes and clarify their overall similarity. Such monoclonal antibodies should also simplify purification of MAO A and facilitate comparative structural, physiological, and genetic studies of MAOs A and B.

We describe here four hybridomas which stably secrete monoclonal antibodies that specifically recognize purified human placental MAO A. Three of the four monoclonal antibodies immunoprecipitate human MAO A but not MAO B from crude mitochondrial extracts, whereas the fourth does not immunoprecipitate either MAO A or MAO B from these extracts. The four antibodies are different, judging from differences in their ability to bind MAO A in different assay systems and to inhibit the enzyme. The existence of unique MAO A-specific epitopes greatly strengthens the hypothesis that MAO A and MAO B are different proteins. A preliminary report of this work has been presented elsewhere (Kochersperger et al., 1984).

Materials and Methods

Purification of MAO A from human placenta. Details of the purification method for MAO A will be reported elsewhere (Weyler and Salach, 1985). Mitochondria were harvested from placentas within 1 to 2 hr of delivery by differential centrifugation and were stored at -70°C . Mitochondria from up to seven placentas were digested with phospholipases A and C, and the digest was centrifuged. The pellet was suspended in buffer and extracted with Triton X-100 (1 mg of detergent/3 mg of protein). After centrifugation, the soluble MAO A activity was partitioned in a two-phase polymer system of dextran and polyethylene glycol. MAO activity was precipitated with 25% polyethylene glycol from the upper phase. Centrifugation gave a pellet which was dissolved in buffer and chromatographed on a Sepharose-DEAE column using salt gradient elution. Fractions with the highest specific activity (assayed spectrophotometrically, using kynuramine from Sigma Chemical Co., St. Louis, MO, as substrate) were pooled and used for the experiments described here.

Immunization of animals. Female BALB/c mice (Texas Inbred Mice Company Breeding Laboratories, Houston, TX) were immunized by a modification of the protocol of Stahl et al. (1980). Basically, 20 or 40 μg of purified human placental MAO A (50/50 emulsion of enzyme in 0.05 M potassium phosphate buffer, pH 7.4, and complete Freund's adjuvant; 0.2 ml total

volume) was administered by multiple routes (including subcutaneous, footpad, and intraperitoneal). The immunization was repeated 2 weeks later with 20 μg of antigen in incomplete Freund's adjuvant. Seven, 8, and 9 days later, each animal was injected intraperitoneally with 20 μg of purified enzyme in sterile saline. On the 10th day, spleen cells were isolated for fusion. Prior to splenectomy, immunized mice were bled from the tail vein to obtain polyclonal anti-MAO A sera which were used as positive controls in enzyme-linked immunosorbent assays (ELISAs) and immunoprecipitation assays.

Generation, subcloning, and storage of hybridomas. Fusion of immune spleen cells to the hypoxanthine-guanine phosphoribosyl transferase-deficient mouse myeloma cell line P3/X63 Ag8 with polyethylene glycol ($M_r = 4000$; American Type Culture Collection, Rockville, MD) was performed according to the method of Kennett et al. (1978). Conditioned media from growing cells were assayed for antibody to human placental MAO A by their ability to immunoprecipitate catalytic activity and to bind purified enzyme bound to microtiter plates (ELISA; see below). Hybridoma cultures of interest were subcloned by limiting dilution, frozen, and stored in liquid nitrogen as previously described (Denney et al., 1982b).

Preparation of ascites fluids. Ascites fluids of hybridoma cultures were produced by injecting 10^7 cells intraperitoneally into Pristane-primed, female BALB/c mice (Kennett et al., 1978).

Immunoprecipitation of MAO A activity. Immunoprecipitation of catalytically active MAOs A and B was performed as described for MAO B by Denney et al. (1982b). Briefly, 50 μl of conditioned media or diluted ascites fluids were mixed with 25 μl of purified human placental MAO A (containing ~ 0.25 to $0.5 \mu\text{g}$ of purified enzyme) diluted in immunoprecipitation buffer (Dulbecco's phosphate-buffered saline (PBS); containing, per liter, 0.2 g of KCl, 0.2 g of KH_2PO_4 , 9 g of NaCl, 1.14 g of Na_2HPO_4 , pH 7.4, plus 1 mg/ml of bovine serum albumin, 0.05% (v/v) Nonidet P40 (Particle Data Laboratories, LTD, Elmhurst, IL)), and incubated for 1 hr at 4°C . Rabbit anti-mouse IgG (20 μl in PBS containing $\sim 80 \mu\text{g}$ of specific antibody; Cappel Laboratories, Cochranville, PA) was added, and 1 hr later 50 μl of a 10% (w/v) suspension of heat-killed, fixed *Staphylococcus aureus* Cowan I bacteria (Pansorbin, Calbiochem-Bioehring, Inc., La Jolla, CA), which had been washed and suspended in immunoprecipitation buffer, was added. After a further 15 min, the bacteria were sedimented at $10,000 \times g$ in a Microfuge (Beckman Instrument Co., Palo Alto, CA), and the supernatant was removed for assay of residual MAO A activity. Pellets were washed twice with 1 ml of immunoprecipitation buffer, resuspended in the same buffer, and assayed for MAO A activity. In screening assays, when conditioned media appeared to remove significant activity from the supernatant, pellets were also assayed for MAO A activity. Immunoprecipitation of ^{125}I -labeled MAO A was performed essentially as described except that samples of supernatants and resuspended pellets were assayed by mixing with liquid scintillation fluid Hydrofluor (National Diagnostics, Somerville, NJ) and counting in an LKB 1217 liquid scintillation counter (LKB Instruments, Gaithersburg, MD) with settings for ^3H . (The corresponding ^3H counting efficiency was 30%.)

Enzyme-linked immunosorbent assay (ELISA). ELISAs were performed as previously described (Denney et al., 1983). For screening assays, 96-well polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with purified human MAO A at a concentration of 1 $\mu\text{g}/\text{ml}$ in borate buffer containing (per liter): 6.2 g of H_3BO_3 , 9.5 g of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, and 9 g of NaCl (pH 8.2) at 37°C overnight. Mouse polyclonal anti-MAO A sera from animals used for fusion bound readily to wells coated in this way, although coating with 4 $\mu\text{g}/\text{ml}$ of MAO A resulted in somewhat higher binding. Samples (150 μl) of conditioned media or ascites fluids diluted as indicated in PBS-Tween (PBS containing 0.05% Tween 20; Sigma Chemical Co.) were added to the microtiter wells and incubated at room temperature for 6 hr. Bound immunoglobulin was detected by adding to each well 150 μl of 1/1000 diluted peroxidase-conjugated goat or sheep anti-mouse IgG (heavy and light chain specific; Cappel Laboratories) in PBS-Tween and incubating 6 hr at room temperature. Bound peroxidase activity was revealed by adding to washed wells 200 μl of peroxidase reaction mix (1 mg/ml of o-phenylenediamine in citric acid buffer, containing (per liter) 1 g of citric acid monohydrate, 1.46 g of Na_2HPO_4 , pH 4.9, and 5 μl of 30% H_2O_2 /25 ml). Peroxidase reactions were stopped after 2 to 5 min by the addition of 50 μl of 1 M H_2SO_4 . Absorbance was measured at 490 nm using a Dynatech model MR-580 Automated ELISA reader (Dynatech Laboratories), and is expressed as absorbance change per minute of reaction.

Assay of MAO activity. MAO activity was assayed by the radiometric assay of Wurtman and Axelrod (1963) at 37°C in 0.1 M potassium phosphate buffer, pH 7.4, as previously described (Denney et al., 1982b). ^{14}C -Serotonin (5-hydroxytryptamine (5-HT), 100 μM , 51 Ci/mol; New England Nuclear, Boston, MA) was used as substrate in many screening assays and in assays of MAO A in human liver. At this concentration, serotonin is primarily oxidized

by MAO A (Johnston, 1968). Tyramine (100 μ M; 55.3 Ci/mol; New England Nuclear) was used as substrate in some screening assays employing purified MAO A. 14 C- β -PEA (10 μ M, 50 Ci/mol; New England Nuclear) was used as a substrate for MAO B assays. At this concentration, β -PEA is primarily oxidized by MAO B (Yang and Neff, 1973). Reaction time was 10 min for MAO A assays and 30 min for MAO B. One unit of oxidizing activity equals 1 nmol/hr, and specific activities are expressed in units per milligram of protein as determined by the method of Lowry et al. (1951).

Effect of antibody binding on MAO A activity. Antibodies from ascites fluids were precipitated three times with 50% saturated ammonium sulfate. Precipitates were suspended in 0.05 M potassium phosphate buffer (pH 7.4) and dialyzed against four changes of the same buffer. Purified MAO A was diluted and dispensed in two equal samples (0.5 μ g) for each antibody tested. Enzyme activities were measured in triplicate 1 hr after addition of 1 μ l of antibody preparation. (Preliminary immunoprecipitation experiments showed that the quantities of MAO A-3C9, A-4F10, A-7B10, and A-7E10 antibodies added were sufficient to precipitate 65.4, 91.4, 96.8, and 100% (respectively) of the recovered activity.) Data from these experiments was subjected to one-way analysis of variance using SPSS (Statistical Package for the Social Sciences) to confirm the existence of treatment effects, followed by contrast analysis (*t* test) to compare the measured activity of each sample with that of the control incubation (buffer alone) or with other samples.

Human platelet MAO B. Samples of human platelet MAO B were generously provided by Drs. N. T. Patel and C. W. Abell. The enzyme was partially purified through the DEAE-Sephacel and ammonium sulfate precipitation steps as previously described (Ansari et al., 1983).

Labeling of MAO A with 125 I. Purified MAO A (500 μ g in 200 μ l of 0.02 M sodium phosphate buffer, pH 7.3) was mixed with 80 μ g of lactoperoxidase (Sigma Chemical Co.) in 80 μ l of phosphate buffer and with 0.4 mCi of carrier-free 125 I (New England Nuclear). The iodination was initiated by adding 20 μ l of 0.09 M H_2O_2 , incubated for 10 min at room temperature, and terminated by adding 800 μ l of 0.02 M phosphate buffer. Radiolabeled protein was precipitated by addition of saturated ammonium sulfate to 50% saturation. The pellet was suspended in 100 μ l of 0.02 M phosphate buffer and dialyzed overnight at 4°C against four changes of the same buffer. The dialysate was centrifuged at 10,000 $\times g$ for 10 min, and the supernatant was used for the immunoprecipitations reported below.

Isolation and extraction of mitochondria. Mitochondria were isolated from human liver and placenta by differential centrifugation in 0.25 M sucrose, as previously described (Denney et al., 1982b). MAO was solubilized from mitochondria by stirring for 20 min at room temperature in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.5% (v/v) Triton X-100 (Bio-Rad Laboratories, Richmond, CA). Insoluble material was removed by centrifugation at 10,000 $\times g$.

SDS-polyacrylamide gel electrophoresis. Samples for electrophoresis were suspended in SDS sample buffer (2% SDS in 0.5 M Tris-HCl, pH 6.8, containing 5% β -mercaptoethanol, 10% glycerol), boiled for 2 min, and spun at 10,000 $\times g$. The supernatants were electrophoresed in a 1.5-mm slab gel according to the method of Laemmli (1970), using a 4% stacking gel and a 12% separating gel (running conditions: 40 mA for 4 hr at room temperature with cooling by circulating tap water). Gels were stained with silver by the technique of Merrill et al. (1981). Gels were photographed using Polaroid type 55 Land film (4 $\times$ 5 inches) and dried overnight at room temperature between sheets of cellophane film (DuPont Chemical Corp., Wilmington, DE). Gels containing radiolabeled proteins were treated with Enlightening according to the manufacturer's directions (New England Nuclear) and dried as described above. Autoradiography was accomplished by exposing Kodak x-ray film to the dried gel at -80°C for 4 to 10 days without an intensifying screen.

Results

Characterization of purified human placental MAO A. MAO A was purified 95-fold from extracts of human placental mitochondria with a 35% recovery. The purified MAO A preparation exhibited several minor bands and a major band with an apparent M_r = 64,000, as judged by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 1). The molecular weight of the major band is in close agreement with previously published values for the molecular weight of MAO A (M_r = 64,000 to 67,000; Callingham and Parkinson, 1979; Brown et al., 1980; Cawthon et al., 1981). The enzyme preparation had an A_{277}/A_{450} absorbance ratio of 14.5 and was capable of oxidizing approximately 29 μ mol of serotonin/hr/mg of protein under our assay conditions.

Figure 1. SDS-polyacrylamide gel of purified MAO A used for immunization and screening. Varying quantities of purified human placental MAO A were reduced and run in SDS-polyacrylamide gels and were silver stained as described under "Materials and Methods." From left to right: Lane 1, chick erythrocyte membrane proteins run as a control, with molecular weights ($\times 10^{-3}$) indicated; lanes 2 to 7, 0.25, 0.375, 0.5, 0.625, 0.75, and 0.875 μ g of purified MAO A, respectively.

Screening of primary hybridomas. Spleen cells from two mice immunized with purified human placental MAO A were fused to the HPRT-deficient myeloma cell line P3/X63 Ag8. Two to 4 weeks after fusion, conditioned medium from wells containing viable cells were tested for antibody to MAO A by immunoprecipitation of catalytic activity and ability to bind MAO A coated at a concentration of 1 μ g/ml in polystyrene microtiter wells (ELISA). These two screening protocols were chosen because immunoprecipitation of MAO A activity is a highly specific assay, whereas ELISA is less specific but relatively fast and reproducible.

Immunoprecipitation was judged positive if the activity in the supernatant was consistently less than 85% of the activity remaining in the supernatant after control immunoprecipitation with P3/X63 Ag8. ELISA was judged positive if the bound peroxidase activity was >2 standard deviations above the mean of the tested medium samples. Of 532 wells screened, media from 3 (0.6%) were judged positive by immunoprecipitation only, 32 (6.0%) were judged positive by ELISA only, 1 (0.2%) was judged positive by both assays, and 496 (93.2%) were judged negative by both assays.

Subclones of these four anti-MAO A hybridomas (12 of 12 from MAO A-3C9, 8 of 9 from MAO A-4F10, 4 of 4 from MAO A-7E10, and 9 of 9 from MAO A-7B10) secreted antibody which had the same pattern of reactivity in the immunoprecipitation assays and ELISAs as the respective primary cultures. In the remainder of this report we use the four antibodies which immunoprecipitate purified catalytically active MAO A (MAO A-3C9, A-4F10, A-7B10, and A-7E10) to characterize the distribution and properties of the respective epitopes on human MAO A and MAO B from different tissues.

ELISA reactivity of MAO A-3C9, A-4F10, A-7B10, and A-7E10. Antibodies MAO A-3C9, A-4F10, and A-7E10 in ascites fluids (not shown) or purified from ascites fluids by ammonium sulfate precipitation exhibited no more binding to microtiter wells coated with MAO A at 1 μ g/ml than did similar preparations of control antibody (SgA-5B11; Woodard et al., 1984) containing antibody to human secretory IgA (Fig. 2A). In contrast, MAO A-7B10 antibody from ascites fluids, conditioned media (not shown), or ammonium sulfate-precipitated preparations gave significant levels of immunoglobulin binding (Fig. 2A). All four anti-MAO A antibodies bound readily to microtiter wells

Figure 2. Reactivity of monoclonal antibodies with purified MAO A by ELISA. Samples of antibodies precipitated from ascites fluids with ammonium sulfate were serially diluted in microtiter wells (150 μ l/well) coated with 1 μ g/ml (A) or 4 μ g/ml (B) of purified MAO A, and bound immunoglobulin detected with peroxidase-conjugated anti-mouse IgG as described under "Materials and Methods." MAO A-3C9 (x-x), MAO A-4F10 (◇), MAO A-7B10 (+--+), MAO A-7E10 (□), and SlgA-6C4 (▲) are shown. Curves in A for MAO A-3C9, A-4F10, A-7E10, and SlgA-6C4 are virtually identical and represent background levels of nonspecific binding.

coated with MAO A at 4 μ g/ml, and highest levels of binding again were observed with MAO A-7B10 (Fig. 2B). These results suggest that the density of MAO A coated on the plastic was critical for detection of binding of MAO A-3C9, A-4F10, and A-7E10, but less so for MAO A-7B10.

Indirect immunoprecipitation of purified MAO A by MAO A-3C9, A-4F10, A-7B10, and A-7E10. Antibodies from ascites fluids were used to determine the proportion of purified MAO A molecules which were recognized by each antibody. Titration of the ascites fluids against 0.25 μ g of 125 I-labeled MAO A indicated that 0.3 to 1.0 μ l of each ascites fluid gave optimum immunoprecipitation of labeled MAO A. Based on this information, ratios of enzyme and ascites fluids were adjusted in subsequent experiments to give optimum precipitation.

Since ascites fluids from MAO A-4F10, A-7E10, and A-7B10 (50 μ l of a 1/100 dilution) immunoprecipitated >93% of the catalytically active, purified MAO A (Table I, Experiment A), epitopes recognized by these antibodies are probably present on most or all catalytically active MAO A molecules. In contrast, ascites fluid from MAO A-3C9

TABLE I
Immunoprecipitation of MAO A activity by ascites fluids containing antibodies MAO A-3C9, MAO A-4F10, MAO A-7E10, and MAO A-7B10^a

Experiment ^b	Antibody Source	Activity Recovered ^c (nmol/hr)	Recovered Activity in Pellet (%)
A	MAO A-3C9	5.7	39
	MAO A-4F10	2.6	93
	MAO A-7E10	2.8	98
	MAO A-7B10	3.4	100
	P3/X63 Ag8	6.2	0
B	MAO A-3C9	2.9	35
	MAO A-4F10	0.6	94
	MAO A-7E10	0.9	100
	MAO A-7B10	1.5	100
	P3/X63 Ag8	1.9	0

^a Samples of purified MAO A were indirectly immunoprecipitated with dilutions of ascites fluids containing anti-MAO A antibodies or conditioned medium from P3/X63 Ag8, and MAO A activity in the supernatants and pellets was assayed with 14 C-serotonin as described under "Materials and Methods."

^b In experiment A, 0.5 μ g of purified MAO A was immunoprecipitated with 50 μ l of 1/100 diluted anti-MAO A ascites fluids or 50 μ l of conditioned medium from P3/X63 Ag8. In experiment B, MAO A activity which remained in the supernatant after immunoprecipitation with MAO A-3C9 ascites fluid (experiment A) was immunoprecipitated with 25 μ l of 1/10 diluted anti-MAO A ascites fluids or 50 μ l of conditioned medium from P3/X63 Ag8.

^c Activity recovered is the sum of the measured activities in the supernatant and pellet after immunoprecipitation.

(at the same dilution) immunoprecipitated only 39% of the MAO A activity in this experiment. To test whether immunoprecipitation of MAO A with MAO A-3C9 resolved MAO A molecules into discrete populations, MAO remaining in the supernatant after immunoprecipitation with MAO A-3C9 was immunoprecipitated a second time with each of the four anti-MAO A antibodies. Results of the second immunoprecipitation show that MAO A-3C9 precipitated only 35% of the recovered activity (Table I, Experiment B), whereas MAO A-4F10, A-7B10 and A-7E10 precipitated all of the MAO A activity as before. In another experiment immunoprecipitation with higher ratios of MAO A-3C9 ascites fluid to MAO A removed from the supernatant >95% of the catalytically active MAO A. These results indicate that the MAO A-3C9 epitope, like those recognized by MAO A-4F10, A-7B10, and A-7E10, occurs on most or all catalytically active MAO A in the purified preparation.

Molecular weight determination of the immunoprecipitated material. The ability of the four antibodies (but none of the 528 others tested) to immunoprecipitate MAO A activity is strong evidence of their specificity for MAO A. As a further demonstration of specificity, 125 I-labeled MAO A was immunoprecipitated, and the immunoprecipitated material was run on an SDS-polyacrylamide gel to determine whether its apparent molecular weight corresponded to that expected for MAO A (M_r = ~64,000; Brown et al., 1980). It is apparent from the autoradiogram of the gel (Fig. 3) that antibodies MAO A-3C9 (Fig. 3, lane 1), MAO A-4F10 (Fig. 3, lane 2), MAO A-7B10 (Fig. 3, lane 3), and MAO A-7E10 (Fig. 3, lane 4) all immunoprecipitated the MAO peptide (M_r = 63,000 to 66,000). Some minor bands were also detected in the immunoprecipitates. These may be fragments of MAO A or contaminants which are associated with some MAO A molecules.

Effect of antibody binding on MAO A activity. The total recovery of MAO A activity after immunoprecipitation (activity in supernatant plus pellet) was significantly lower for MAO A-specific antibodies than for nonspecific antibodies (see Table I). In order to determine whether this observed reduction in activity was a direct result of antibody binding, ammonium sulfate-purified antibodies from ascites

Figure 3. Autoradiograph of SDS-polyacrylamide gel of ^{125}I -labeled MAO A after immunoprecipitation with monoclonal antibodies. Samples of ^{125}I -labeled human placental MAO A ($\sim 1 \mu\text{g}$; 137,000 cpm) were immunoprecipitated with 200 μl of conditioned medium from hybridoma cultures. Labeled material was eluted from the pellets by boiling in SDS-sample buffer and electrophoresed as described under "Materials and Methods." Gel lanes contained 50 μl of material precipitated with the following antibodies: lane 1, MAO A-3C9 (6,000 cpm); lane 2, MAO A-4F10 (10,000 cpm); lane 3, MAO A-7B10 (15,000 cpm); lane 4, MAO A-7E10 (12,000 cpm); lane 5, P3/X63 Ag8 (control precipitation; 2,000 cpm); lane 6, MAO A (no precipitation, 200,000 cpm).

fluids were incubated with purified MAO A, and enzyme activities were measured before and after incubation.

The results (Table II) show that incubation with MAO A-7E10 and A-3C9 resulted in marked inhibition (29% and 73%, respectively, $p < 0.0001$), compared to control incubations with buffer alone or with non-MAO A-binding antibodies MAO B-1C2 or SC-6C4. The slight inhibition of MAO A observed with MAO A-7B10 or A-4F10 was not significant ($p < 0.16$). Although MAO A-7B10 did not inhibit catalytic activity in these experiments, other experiments showed that when ammonium sulfate-purified MAO A-7B10 precipitated $>90\%$ of the activity from the supernatant, the total activity recovered was generally less than 50% of that recovered with non-MAO A-specific antibodies. Non-specific factors (besides inhibition due to antibody binding) apparently reduce the recovery of activity after immunoprecipitation.

Selectivity of antibodies for human MAO A and MAO B. We tested the four anti-MAO A antibodies for their ability to react with human MAO A and/or MAO B from crude extracts to see whether the determinants recognized by MAO A-3C9, A-4F10, A-7B10, or A-7E10 on the purified MAO A were expressed on MAO A or MAO B from other tissues. To help ensure maximal precipitation, crude extracts were diluted so that the amount of MAO A activity immunoprecipitated corresponded to $<0.5 \mu\text{g}$ of purified MAO A.

Detergent extracts of human placental mitochondria (MAO A only) were immunoprecipitated with MAO A-3C9, A-4F10, A-7B10, and A-7E10 from ascites fluid. Most recovered MAO A activity was found in the pellets after immunoprecipitation with MAO A-4F10, A-7B10, and A-7E10 (81%, 97%, and 77%, respectively; Table III). Little or no MAO A activity was recovered in the pellets after immunoprecipitation with MAO A-3C9 (Table III). As expected, MAO B-1C2 did not immunoprecipitate MAO A.

Antibodies were then tested for their ability to react with catalytically active human platelet MAO B. Human platelets contain MAO B but not MAO A. None of the four anti-MAO A antibodies immunoprecipitated MAO B activity from platelet extracts (Table III). In contrast, MAO B-1C2, as has been previously shown, efficiently immunoprecipitated MAO B activity from human platelets.

Antibody from conditioned media from three of the four anti-MAO A hybridomas (MAO A-4F10, A-7B10, and A-7E10) immunoprecipitated MAO A but not MAO B activity from detergent extracts of human liver mitochondria (Table IV). The data indicate that there is no detectable cross-reaction between the anti-MAO A antibodies

TABLE II
Effect of antibody binding on MAO A activity^a

Antibody Source	MAO A Activity ^b (nmol/hr)	Inhibition ^c (%)
MAO A-3C9	0.5 ± 0.04^d	73
MAO A-4F10	1.6 ± 0.12	16
MAO A-7B10	1.6 ± 0.25	16
MAO A-7E10	$1.3 \pm 0.27^{d,e}$	29
MAO B-1C2	2.0 ± 0.22	0
SC-6C4	1.8 ± 0.17	0
None (buffer only)	1.8 ± 0.16	(0)

^a Purified MAO A (0.5 μg) was incubated for 1 hr at 4°C with 1 μl of ammonium sulfate-purified antibody from the indicated ascites fluids.

^b MAO A activity was measured in triplicate on two samples for each antibody both before and after incubation with antibody. Values represent the mean $\pm$ SD. The mean activity measured in tubes before incubated was 2.57 ± 0.14 .

^c Calculated as $100 \times (\text{activity after antibody}/\text{activity after buffer})$.

^d Differed from buffer control ($p < 0.0001$).

^e Differed from MAO A-7B10 and -4F10 ($p < 0.03$).

TABLE III
Immunoprecipitation of human placental MAO A and human platelet MAO B from crude extracts^a

Antibody Source	Placental MAO A		Platelet MAO B	
	Activity Recovered ^b (nmol/hr)	Recovered Activity in Pellet (%)	Activity Recovered ^b (nmol/hr)	Recovered Activity in Pellet (%)
MAO A-3C9	6.9	2	5.1	<1
MAO A-4F10	1.5	81	4.7	<1
MAO A-7B10	2.2	97	4.5	0.2
MAO A-7E10	1.5	77	6.4	0.1
MAO B-1C2	8.5	2	2.4	97
P3/X63 Ag8	9.0	0	ND ^c	ND

^a Ascites fluids containing the indicated antibodies (50 μl diluted 1/100) or conditioned medium from P3/X63 Ag 8 (50 μl) were used to immunoprecipitate MAO A activity from 10 μg total protein of a Triton X-100 extract of human placental mitochondria or MAO B activity from 50 μg total protein of DEAE-purified human platelet MAO B. MAO activity left in the supernatants and pellets was assayed as described under "Materials and Methods."

^b Activity recovered is the sum of the measured activities in the supernatant and pellet after immunoprecipitation.

^c ND, not determined.

TABLE IV
Immunoprecipitation of MAO A and B activity from extracts of human liver mitochondria^a

Antibody Source	MAO A		MAO B	
	Activity Recovered ^b (nmol/hr)	Recovered Activity in Pellet (%)	Activity Recovered (nmol/hr)	Recovered Activity in Pellet
MAO A-3C9	5.4	0.9	3.3	0.0
MAO A-4F10	2.4	52	4.6	0.3
MAO A-7B10	2.5	93	4.3	1.1
MAO A-7E10	1.7	49	2.9	0.2
MAO B-1C2	4.7	0.8	2.7	99
HY medium	5.7	0.1	3.2	0.0

^a Samples of a Triton X-100 extract of human liver mitochondria (50 μ g total protein) was immunoprecipitated with conditioned or control medium from the indicated sources and the resulting supernatants and pellets assayed as described under "Materials and Methods." Data are expressed as percentage of recoverable activity in the pellet.

^b Activity recovered is the sum of the measured activities in the supernatant and pellet after immunoprecipitation.

and liver MAO B. Immunoprecipitation of MAO A by MAO A-4F10 and A-7E10 was incomplete in this experiment due to the addition of limiting amounts of antibody. Other experiments using higher concentrations of these antibodies from ascites fluids showed virtually complete precipitation of MAO A activity from liver mitochondrial extracts. In contrast, MAO A-3C9 (at all antibody concentrations tested) did not immunoprecipitate significant MAO A or MAO B activity from these liver extracts (Table IV).

Discussion

We report here the isolation of four hybridoma cell lines which secrete monoclonal antibodies to MAO A. Specificity of these antibodies for MAO A was demonstrated by their ability to precipitate MAO A catalytic activity coincident with a peptide having an apparent molecular weight on SDS-polyacrylamide gels that corresponds with that of MAO A. The four antibodies differ in the characteristics of their binding to the MAO A molecule; they differ in their abilities to immunoprecipitate MAO A (Table I), to inhibit MAO A activity (Table II), and to bind to MAO A coated at low density in microtiter wells (Fig. 2). Preliminary competition experiments (not shown) showed that a 10,000-fold excess of either unlabeled MAO A-7B10 or MAO A-3C9 IgG from ammonium sulfate-precipitated ascites fluids completely blocked binding of both [³⁵S] methionine labeled MAO A-7B10 and A-3C9 to human placental mitochondria, whereas equivalent excesses of unlabeled MAO A-4F10 and A-7E10 did not significantly block binding of either labeled MAO A-7B10 or MAO A-3C9. These data suggest that MAO A-3C9 and A-7B10 recognize the same or adjacent epitopes on MAO A and that this epitope is distinct from epitopes recognized by either MAO A-4F10 or A-7E10. We conclude that these four antibodies recognize at least two unique epitopes on MAO A.

The most important result obtained was that three of the four anti-MAO A antibodies (MAO A-4F10, A-7B10, and A-7E10) did not give any detectable immunoprecipitation of human MAO B from platelets or liver under conditions where human MAO A from placenta or liver was efficiently immunoprecipitated. The fourth antibody, MAO A-3C9, could not be tested by immunoprecipitation for its ability to discriminate MAOs A and B because it immunoprecipitated MAO A in crude preparations poorly. However, MAO A-3C9 appears to bind specifically to MAO A, since it competes for binding to MAO A with MAO A-7B10, as indicated above. Furthermore, like MAO A-4F10, A-7B10, and A-7E10, MAO A-3C9 appears to stain MAO A by immunofluorescence in histological sections of human placental tissue, but not MAO B in smears of human platelets (L. Thorpe, K.

Westlund, L. Kochersperger, C. Abell, and R. M. Denney, submitted for publication). The specificity of the antibodies described here for MAO A complements that of MAO B-1C2, which has previously been shown to immunoprecipitate MAO B but not MAO A from extracts of mitochondria from human brain, kidney, liver, and lung (Denney et al., 1983). These studies demonstrate conclusively that MAOs A and B are immunologically distinct, and confirm the existence of at least two (and possibly more) MAO A-specific epitopes (Powell and Craig, 1977; Craig et al., 1982).

Polyclonal antisera which have been used to investigate the immunological relationship of MAOs A and B, have given conflicting results. It appears that polyclonal antisera elicited to MAO purified from tissues which express either MAO A or MAO B, but not both, exhibit considerable type specificity (McCauley and Racker, 1973; Powell and Craig, 1977; Craig et al., 1982; Pintar et al., 1983). Our results with polyclonal antisera (not shown) elicited in mice to purified human placental MAO A agree with this. Our anti-MAO A sera appear to be quite specific for MAO A but, at high concentrations, also appear to partially immunoprecipitate ³H-pargyline-labeled human platelet MAO B. The high type specificity of available monoclonal antibodies and the low titers of cross-reacting antibodies in polyclonal sera suggest that only a low proportion of such cross-reacting antibodies are elicited in response to pure MAO A or MAO B. The cross-reacting antibodies in polyclonal antisera could be elicited to immunochemically related portions of MAOs A and B or to trace amounts of MAO B in the purified MAO A preparation.

The epitopes on MAO A studied here are physically associated with most or all of the soluble MAO A molecules in the purified preparation. The simplest interpretation of the association of the epitopes with most or all MAO A activity is that the epitopes recognized by the four antibodies are present on all MAO A molecules, which are believed to be dimers. However, MAO A subunits readily associate in the absence of detergents to form high molecular weight aggregates (Brown et al., 1980). Since we did not examine the state of aggregation of MAO A under the conditions of our immunoprecipitation assays, it is possible that each MAO A-specific epitope studied here is present on a subset of MAO A molecules, and that at least one of these epitope-containing molecules is present in most or all aggregates containing MAO A catalytic subunits. It has been proposed that MAOs A and B can associate to form a mixed function, high molecular weight, lipid-containing complex (White and Tansik, 1979). However, since MAO A and B activities of human liver are easily separable by immunoaffinity chromatography on Sepharose-MAO B-1C2 columns (Denney et al., 1982a) or by immunoprecipitation with either anti-MAO A- or MAO B-specific antibodies (Table IV), MAO A and B catalytic subunits did not coexist in complexes in human liver mitochondrial extracts under the conditions of our experiments.

Epitopes recognized by three of the four antibodies (MAO A-4F10, A-7B10, and A-7E10) were also present on all (>90%) of the catalytically active MAO A from extracts of human liver and placental mitochondria, but not on catalytically active MAO B from extracts of human platelets or human liver mitochondria (Tables II and IV). MAO A-3C9 did not immunoprecipitate MAO A or MAO B from these crude extracts. This may reflect its apparently lower affinity for MAO A. In contrast, MAO A-3C9 does bind to unextracted human placental mitochondria (not shown), suggesting that the epitope recognized by MAO A-3C9 might be masked or altered by detergent-protein, protein-protein, or lipid-protein interactions in these detergent-solubilized extracts.

The immunoprecipitation of MAO A activity by the anti-MAO A monoclonal antibodies clearly demonstrates their specificity for MAO A. However, the proportion of recoverable catalytic activity precipitated by the antibodies may not yield a quantitative estimate of the proportion of MAO A molecules precipitated. For example, unpurified ascites fluids (used in most immunoprecipitations reported here), including those which contained no MAO A-binding antibody, appeared to have variable effects on catalytic activity (data not shown),

presumably because they contained variable amounts of inhibitory and/or stimulatory factors, other than immunoglobulin. In addition, the catalytic activity recovered after immunoprecipitation was not quantitative, even for MAO A-7B10, an antibody which binds MAO A without significantly inhibiting it (Table II). Nonquantitative recoveries of activity in pellets after immunoprecipitation with specific antibodies may have resulted from mechanical losses, damage to or elution of enzyme from immunoprecipitates during washing of the pellets, or steric hindrance of substrate access to the catalytic site in the immunoprecipitates by secondary reagents (anti-mouse IgG and *S. aureus*). Because recovery of catalytic activity in the supernatant after immunoprecipitation was not affected by the same factors (i.e., inhibition by antibody, mechanical losses, or steric hindrance) as was enzyme recovered in the pellet, the values reported here for percentage of catalytic activity precipitated probably underestimate somewhat the proportion of enzyme molecules bound by antibody. Despite complications in the quantitation of the immunoprecipitation by measuring catalytic activity, we can still conclude that MAO A-3C9, A-4F10, A-7B10, and A-7E10 immunoprecipitate virtually all of the purified MAO A activity from the supernatant.

The MAO A-specific antibodies described here and the MAO B-specific antibody described previously provide valuable reagents for studies of the human monoamine oxidases. MAO B-1C2-Sepharose columns can be used to separate MAO A and MAO B from human liver, and they produce MAO B suitable for structural studies (Denney et al., 1982a), although conditions for eluting catalytically active MAO B from these columns have not yet been found. Catalytically active MAO B-1C2 · MAO B complexes can be purified on Sepharose-protein A columns (Patel et al., 1984). The anti-MAO A monoclonal antibodies may be useful in immunoaffinity purification of MAO A for comparative structural studies of MAOs A and B. It is also possible that these antibodies may facilitate attempts to clone the gene(s) for human MAO A and MAO B, thereby permitting direct comparison of MAO A and B structure. Monoclonal antibodies may also prove useful in studies of the regulation of MAO A synthesis and its translation and insertion into the outer mitochondrial membrane. Preliminary evidence from our laboratory demonstrates that these antibodies do not recognize mouse MAO A or B and, therefore, may complement MAO B-1C2 in ongoing studies of the genetic regulation of human MAO in mouse × human somatic cell hybrids (Kochersperger et al., 1983; Kochersperger and Denney, 1984). In addition, preliminary immunocytochemical evidence from studies with the anti-MAO A antibodies and MAO B-1C2 (L. Kochersperger, E. Parker, M. Siciliano, G. Darlington, and R. Denney, submitted for publication) indicate that MAO A and MAO B have entirely different cellular distributions in human and monkey brain, consistent with the view that their physiological roles are highly specialized and completely different. The antibodies will therefore provide important clues to the physiological roles of the two enzymes in controlling levels of biogenic amines in brain and peripheral tissues, and in activating neurotoxins such as MPTP. It is likely that these new data will clarify how regulation and/or alteration of the activity of MAOs A or B might be related to psychiatric, neurologic, or other diseases.

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