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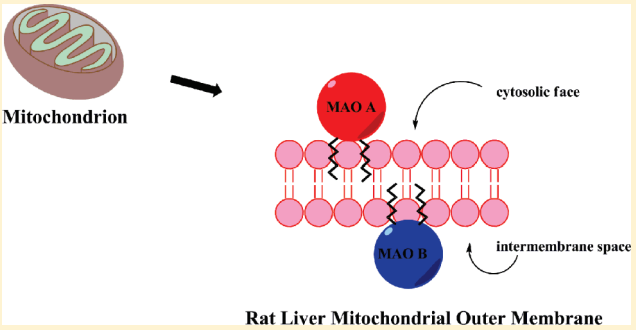
Topological Probes of Monoamine Oxidases A and B in Rat Liver Mitochondria: Inhibition by TEMPO-Substituted Pargyline Analogues and Inactivation by Proteolysis

Jin Wang and Dale E. Edmondson*

Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322, United States

Supporting Information

ABSTRACT: TEMPO-substituted pargyline analogues differentially inhibit recombinant human monoamine oxidase A (MAO A) and B (MAO B) in intact yeast mitochondria, suggesting these membrane-bound enzymes are located on differing faces of the mitochondrial outer membrane [Upadhyay, A., and Edmondson, D. E. (2009) *Biochemistry 48*, 3928]. This approach is extended to the recombinant rat enzymes and to rat liver mitochondria. The differential specificities exhibited for human MAO A and MAO B by the *m*- and *p*-amido TEMPO pargylines are not as absolute with the rat enzymes. Similar patterns of reactivity are observed for rat MAO A and B in mitochondrial outer membrane preparations expressed in *Pichia pastoris* or isolated from rat liver. In intact yeast mitochondria, recombinant rat MAO B is inhibited by the pargyline analogue whereas MAO A activity shows no inhibition. Intact rat liver mitochondria exhibit an inhibition pattern opposite to that observed in yeast where MAO A is inhibited and MAO B activity is unaffected. Protease inactivation studies show specificity in that MAO A is sensitive to trypsin whereas MAO B is sensitive to β-chymotrypsin. In intact mitochondrial preparations, MAO A is readily inactivated in rat liver but not in yeast upon trypsin treatment and MAO B is readily inactivated by β-chymotrypsin in yeast but not in rat liver. These data show MAO A is oriented on the cytosolic face and MAO B is situated on the surface facing the intermembrane space of the mitochondrial outer membrane in rat liver. The differential mitochondrial outer membrane topology of MAO A and MAO B is relevant to their inhibition by drugs designed to be cardioprotectants or neuroprotectants.



The known age-related increases in the levels of expression of monoamine oxidase B (MAO B) in neuronal tissue[1] and monoamine oxidase A (MAO A) in heart[2] have been implicated in neurological[3] and cardiovascular disorders.[4] Design of highly specific reversible inhibitors for each enzyme that could serve as neuroprotectants and cardioprotectants has been and is currently receiving an increased level of attention. It is known that MAO A and MAO B levels vary among different tissues.[5] In all cases, both enzymes are dimeric[6] and found tightly bound to the outer membrane of the mitochondrion via C-terminal transmembrane helices as well as undetermined membrane interactions with the core polypeptide chain.[7,8]

In spite of considerable information in the literature about the structures of MAO A[8,9] and MAO B,[7] their respective substrate and inhibitor specificities, and expression, there is little knowledge about the membrane topology of either enzyme. Early work to address this issue utilized polyclonal antibodies[10] and susceptibility to proteolysis.[11] The results of these studies resulted in conflicting conclusions about the membrane orientations of MAO A and MAO B. Knowledge of the outer membrane topology of MAO A and MAO B is an important issue relative to selective inhibitor design. Although the mitochondrial outer membrane is classically thought to be permeable to $\leq$6 kDa molecules,[12] more recent work has demonstrated this permeability is highly controlled.[13] Therefore, the assumption that outer membrane permeability would present no obstacles to MAO inhibitors (if they were required to traverse the outer membrane) to bind to the active site of either enzyme may not be valid. Alternatively, both enzymes may be oriented toward the cytosolic face of the outer membrane, and therefore, the issue of transport of inhibitors across the outer membrane becomes moot.

Previous published work from this laboratory[14] has shown that TEMPO-substituted pargyline (ParSL-1−3, structures in Figure S1 of the Supporting Information) analogues exhibit differential reactivities with human MAO A and MAO B depending on whether the TEMPO moiety is in the *meta* (ParSL-3) or *para* (ParSL-2) amide linkages with the benzene ring, whereas ParSL-1 inactivates either enzyme.[14] It was also demonstrated that, in intact mitochondria isolated from the expression strain of *Pichia pastoris*, human MAO B is readily inactivated by ParSL-1 whereas

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human MAO A is unreactive. Similar experiments with intact human placental mitochondria demonstrated MAO A is readily inactivated by ParSL-1. These results were interpreted to suggest that recombinant human MAO A is oriented on the surface facing the intermembrane space of the *Pichia* outer membrane and on the cytosolic face of human placental mitochondria, whereas recombinant human MAO B faces the cytosolic side of the *Pichia* mitochondrial outer membrane.

These results provide the basis for the applicability of these pargyline analogues as well as proteolysis studies for probing the MAO topology in intact mitochondria. In this paper, we extend this approach to rat liver MAO A and MAO B. The rat is experimentally more accessible for conducting these studies because tissue samples are readily available. It is known that differences in inhibitor sensitivities exist between the rat and human enzymes;[15,16] therefore, comparative inhibition studies are reported for purified and membrane-bound forms of recombinant rat MAO A and MAO B and compared with those using rat liver membrane preparations. Proteolysis studies of rat MAO A and MAO B are also presented and compared with previously published studies.[11] The results of the inhibition and proteolysis studies presented for recombinant and rat liver MAO A and B support the conclusion that rat liver MAO A is located on the cytosolic face of the mitochondrial outer membrane whereas MAO B is bound to the surface facing the intermembrane space. The significance of these findings is discussed with regard to MAO inhibitor design.

EXPERIMENTAL PROCEDURES

Materials. The detergent β-octyl glucopyranoside was obtained from Anatrace Inc., and reduced Triton X-100 was purchased from Fluka. Percoll was obtained from Amersham Biosciences. The TEMPO-substituted pargyline analogues (ParSL-1−3) were synthesized by A. Upadhyay in this laboratory as described previously.[14] All other chemicals used in this work were purchased from Sigma-Aldrich.

Expression and Purification of Rat MAO. Recombinant rat liver MAO A and MAO B were expressed and purified in *P. pastoris* strain KM71 as described previously.[17,18] The purified enzymes were stored in 50 mM potassium phosphate containing 50% (w/v) glycerol and 0.8% (w/v) β-octyl glucopyranoside (pH 7.2) at −20 °C. It should be noted that d-amphetamine, a reversible MAO A inhibitor used for stabilizing rat MAO A enzyme during purification, was removed prior to all kinetic measurements. All determinations of protein concentration were performed using the Biuret procedure.

Isolation of *P. pastoris* Mitochondria and Mitochondrial Outer Membranes. Intact mitochondria from *P. pastoris* cell paste were isolated by enzymatic disruption of the cell walls using zymolyase followed by homogenization and sequential centrifugations.[19] Purified intact mitochondria were resuspended in 0.6 M mannitol and 10 mM Tris-HCl (pH 7.4) to a final concentration of 10 mg of protein/mL. The integrity of the isolated mitochondria was determined by measuring the rate of NADH oxidation following the rate of O_2 uptake with an oxygen electrode relative to a control sample of disrupted mitochondria.[14] The mitochondrial outer membrane (MOM) was isolated by osmotic shock and mild sonication of intact mitochondria followed by centrifugation in a 30−55% (w/v) sucrose density gradient in 10 mM Tris-HCl buffer (pH 7.4) at 20000 rpm (Beckman SW-28 rotor) for 15 h. MOM pellets were suspended in 10 mM Tris-HCl buffer containing 10% (v/v) glycerol at a protein concentration of 10 mg/mL and kept frozen in small aliquots at −80 °C until they were used.

Isolation of Mitochondria from Rat Liver. Intact mitochondria from rat MAO liver were isolated by following a published method.[20] Briefly, one Sprague-Dawley rat was sacrificed, and the liver (20 g) was perfused in 150 mM NaCl to remove any residual blood. The liver was then minced and homogenized [10% (w/v)] in ice-cold buffer containing 230 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EGTA, 0.1% BSA, 1 mM PMSF, 10 mM NaF, 1 mM sodium pyrophosphate, and 4 mM β-glycerophosphate (pH 7.4). Cell debris was removed by centrifugation twice at 600*g* for 5 min. The supernatant was collected and centrifuged at 10300*g* for 10 min. The crude mitochondria were further purified by centrifugation (111406*g* for 30 min) in 30% (w/v) Percoll in the buffer described above. Intact mitochondria were broken after a 1:1 dilution in cold hypotonic buffer [20 mM sucrose, 5 mM MOPS, 1 mM $MgCl_2$, 5 mM KH_2PO_4, and 0.5 mM EGTA (pH 7.4)] followed by vortexing for 5 min.

Measurement of Inhibition. MAO A activity in rat liver mitochondrial preparations using serotonin as a substrate was assayed polarographically at 25 °C in 50 mM potassium phosphate buffer (pH 7.5) by following the rate of O_2 uptake. All other MAO activity assays were performed by monitoring the rate of product formation with time using a Perkin-Elmer Lambda 2 spectrophotometer at 25 °C. Assays of purified rat MAO A and MAO B activity were conducted in 50 mM potassium phosphate buffer (pH 7.5) containing 0.5% (w/v) reduced Triton X-100 using kynuramine or benzylamine as substrates, respectively.[21,22] 3-(2-Aminomethyl)pyridine (3-AmMePy) was used as a MAO B-selective substrate[23] in the Amplex Red−peroxidase coupled assay in rat liver mitochondrial preparations. One unit of catalytic activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of product/min.

Inhibition constants (K_i values) of purified rat MAO A and MAO B with the ParSL analogues were determined by measuring alterations in K_m values for substrates in the presence of four different inhibitor concentrations. The rates of inactivation of MAO in purified enzymes, in intact and/or broken mitochondria, or in MOM preparations were determined by measuring the loss of activity over time upon incubation with 1 mM ParSL analogues at room temperature.

Inhibition of MAO activity by protease treatment of recombinant MOM-bound rat MAO A and B and of rat liver mitochondria was conducted with either trypsin or β-chymotrypsin using a concentration ratio of 1:5 (w/w) of protease to protein at room temperature. Samples were taken at various time periods; protease activity was terminated by the addition of 1 mM PMSF (final concentration), and MAO activities were determined as described above.

RESULTS

Inhibition of Recombinant Rat MAO by Pargyline and Its TEMPO Analogues. To determine whether rat MAO A and MAO B exhibit similar specificities with the TEMPO-substituted pargyline analogues as observed with the human enzymes, the inhibition constants of ParSL-1−3 for purified recombinant rat enzyme preparations were determined. All of the TEMPO-conjugated pargyline analogues function as competitive inhibitors of rat MAO A and B. As shown in Table 1, these pargyline analogues bind to rat MAO A with K_i values ranging from 65 to 165 μM and to rat MAO B with K_i values ranging from 32 to 250 μM. ParSL-1 and ParSL-2 inhibit human MAO B with K_i values of 22

and 15 μM, respectively, with only ParSL-1 demonstrating binding to human MAO A (K_i = 212 μM).[14] ParSL-3 weakly binds to human MAO A (K_i = 268 μM) and exhibits no observable inhibition with human MAO B.[14] The data demonstrate differences in the behavior of the rat enzymes as compared with the human enzymes upon interaction with these pargyline analogues. This differential behavior is also observed in comparisons of rates of inactivation of rat and human MAO A and MAO B.

The results in Figure 1 and Table 2 show that purified and membrane-bound rat MAO A are inactivated by ParSL-1 3.7- and 3-fold more slowly, respectively, than rat MAO B with ParSL-1. Comparable rates of inhibition are observed upon inactivation of either enzyme with ParSL-2 and ParSL-3, although ~40% MAO B activity was lost upon inactivation by ParSL-3, while MAO A exhibits 40% residual activity upon inactivation by ParSL-2. These kinetic results contrast with the behaviors exhibited by purified human enzymes[14] where no inhibition of MAO A is observed with ParSL-2 and MAO B is resistant to inactivation by ParSL-3. Thus, the rat enzymes do not exhibit the high level of specificity for these analogues as observed with the human enzymes. The apparent plateau of rat MAO B inactivation with ParSL-3 to a 50% level of activity also differs from the behavior observed with other analogues and with the human enzyme. Inactivation rates observed with recombinant MOM-bound rat MAO A are similar to those observed with the purified enzyme, the exception being that inactivation by ParSL-2 plateaus at ~50% while other analogues totally inactivate the membrane-bound form of the enzyme.

Table 1. Competitive Inhibition Constants for Inhibition of Purified Recombinant Rat MAO A and MAO B Activities by ParSL Analogues

	K_i (μM)	
inhibitor	MAO A	MAO B
ParSL-1	65.6 ± 4.7	32.2 ± 2.3
ParSL-2	165.9 ± 13.2	83.8 ± 4.0
ParSL-3	125.0 ± 7.4	251.3 ± 14.2

Incubations of intact yeast mitochondria containing the individually expressed recombinant enzymes demonstrate a pattern of inhibition different from that observed with the purified enzymes. Only ~20% inhibition of MAO A activity is observed when intact *Pichia* mitochondria are incubated with 1 mM ParSL-3, whereas essentially complete inhibition is observed with broken mitochondria or MOM (Figure 2A). In contrast, rat MAO B in intact yeast mitochondria is completely inhibited by 1 mM ParSL-2 as observed with broken mitochondria and MOM (Figure 2B).

Thus, recombinant rat MAO A and MAO B in *Pichia* exhibit reactivity patterns similar to those of the ParSL analogues as observed with the human enzymes in this yeast organelle.[14] The data suggest that rat MAO A is unreactive to the TEMPO inhibitor because of its membrane orientation toward the intermembrane face of the MOM while rat MAO B (which is the case for the human enzyme) is oriented to the cytosolic face with unimpeded reactivity with this inhibitor. It should be noted that pargyline functions as an inhibitor with either enzyme in intact mitochondria, which suggests the inability of ParSL-3 to inhibit MAO A is due to the fact that it is unable to be transported across the mitochondrial outer membrane into the intermembrane space between outer and inner membranes.

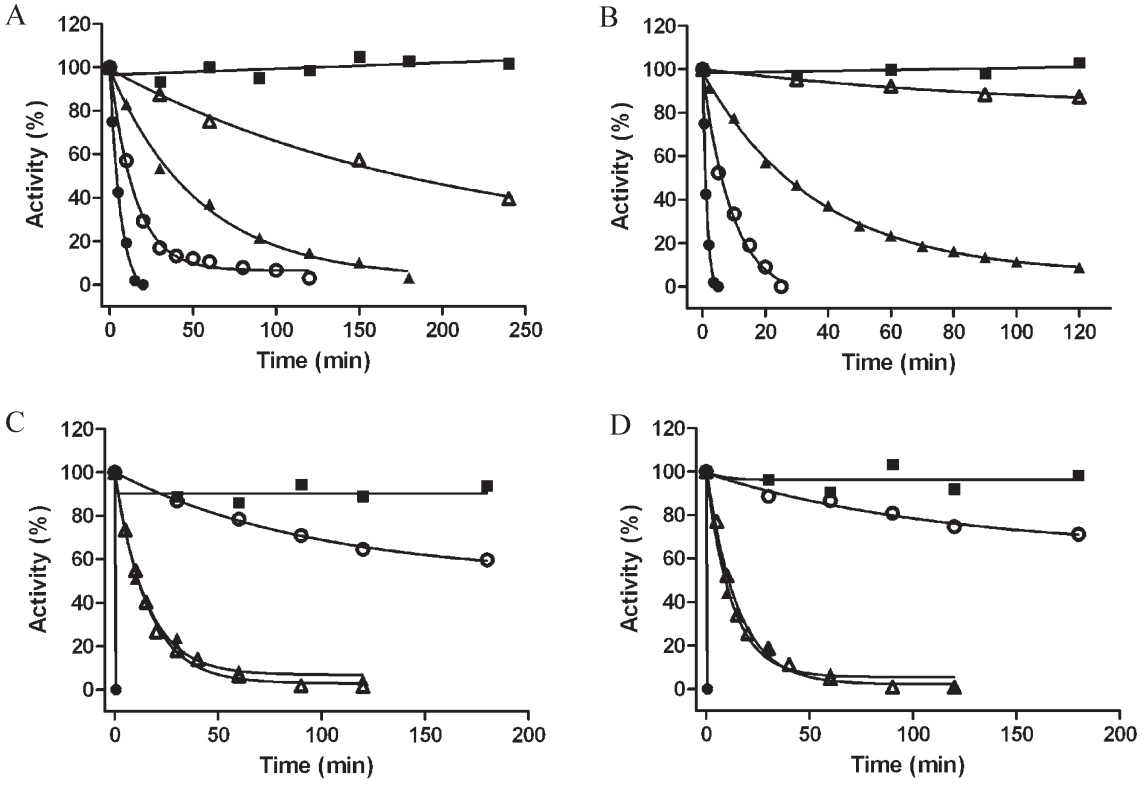

Figure 1. Rates of inhibition of purified rat MAO A (A), MOM-bound rat MAO A (B), purified rat MAO B (C), and MOM-bound rat MAO B (D) with 1 mM pargyline (●), ParSL-1 (▲), ParSL-2 (△), and ParSL-3 (○). The solid lines are exponential fits to the data for a pseudo-first-order rate of decay. The control activities without inhibitor are shown (■). Rate constants for inhibition are listed in Table 2.

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Table 2. Apparent Pseudo-First-Order Rate Constants [$k_{inactivation}$ (s^{-1})] for Inhibition of Recombinant Rat MAO A and MAO B Activities by Pargyline and by TEMPO-Substituted Pargyline Analogues for both Detergent-Purified and MOM-Bound Forms

inhibitor	MAO A (purified)	MAO A (MOM)	MAO B (purified)	MAO B (MOM)
pargyline	9.4 ± 1.1	44.8 ± 6.2	too fast to measure	too fast to measure
ParSL-1	1.1 ± 0.1	1.62 ± 0.06	4.0 ± 0.6	4.8 ± 0.8
ParSL-2	0.3 ± 0.02	0.15 ± 0.04	3.8 ± 0.2	4.1 ± 0.3
ParSL-3	4.0 ± 0.2	6.3 ± 0.9	0.6 ± 0.06	0.54 ± 0.24

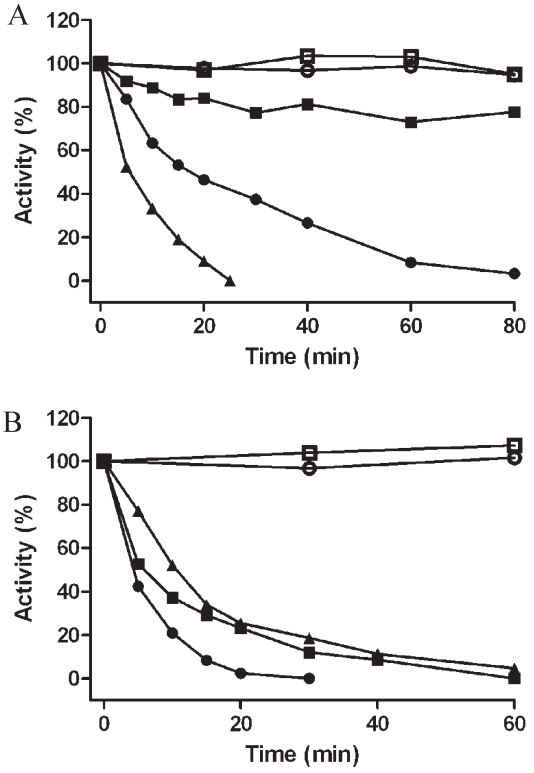

Figure 2. Comparison of the time dependence of rat MAO A inhibition by ParSL-3 (A) and rat MAO B inhibition by ParSL-2 (B) in intact mitochondria (■), broken mitochondria (●), and MOM (▲) of *P. pastoris*. Data for intact mitochondria without ParSL-3 and ParSL-2 treatment and broken mitochondria are shown (□ and ○, respectively). The specific activities of rat MAO A and B MOM preparations are 12.4 and 31.4 milliunits/mg, respectively.

MAO Inhibition in Intact Rat Liver Mitochondria. With this background of MAO reactivities in both purified and membrane-bound forms of the recombinant system, we isolated intact mitochondria from rat liver to ask whether MAO A and MAO B exhibit behaviors consistent with orientations toward the cytosolic face of the MOM or whether they might face toward the intermembrane space. Because rat liver mitochondria contain both MAO A and MAO B at approximately equal levels,[5] isozyme-specific substrates were used to selectively assay MAO A or MAO B. Serotonin oxidation was used for MAO A because its rate of oxidation by MAO B is much lower.[5] Previous work in our laboratory has shown that 3-(2-aminomethyl)pyridine (3-AmMePy) is a good substrate for MAO B but is poorly oxidized by MAO A.[23] The data in panels A and C of Figure 3 show that rat liver MAO A is readily inactivated upon incubation with 1 mM ParSL-3 in both intact and broken mitochondria while only 20% of serotonin oxidase activity is inactivated by ParSL-2. This behavior with ParSL-2 is similar to that observed with recombinant yeast MOM MAO A (Figure 1B). In contrast, when intact mitochondria are assayed for MAO B (Figure 3B), incubation with neither ParSL-2 nor ParSL-3 results in any observable inactivation. Disruption of the mitochondria results in the appearance of sensitivity of rat MAO B to ParSL-2 inhibition (Figure 3D), which was more effective than ParSL-3 [also observed with purified recombinant enzymes (Figure 1A,C)].

Protease Inhibition as an Additional Probe of Membrane Orientation of Rat MAO A and MAO B. An additional probe of membrane orientation was used for comparison with the inhibition data with the TEMPO-pargyline analogues presented above. Buckman et al.[11] demonstrated that human and rat MAO A activity was sensitive to treatment with trypsin. This observation is confirmed in this study using yeast MOM particles containing rat MAO A. When trypsin inactivation studies were performed with yeast MOM containing rat MAO B, no observable inactivation occurs within the time frame observed for MAO A. The data in Figure 4 show that yeast recombinant MOM-bound rat MAO A is sensitive to trypsin whereas β-chymotrypsin specifically inactivates rat MAO B. Therefore, although the two enzymes are ~70% identical in sequence and the human enzymes exhibit a high degree of structural identity, there is a marked difference in sensitivity to inactivation by proteolysis on trypsin and β-chymotrypsin treatment.

These differing reactivities to protease treatment were used to provide additional insights into the MOM topology of rat MAO A and MAO B in intact rat liver mitochondria. The data in Figure 5 show that β-chymotrypsin treatment of rat liver mitochondria (estimated to be 70% intact by NADH oxidase activity) results in an only 30% loss of MAO B activity. In contrast, >90% of MAO A activity is lost upon incubation of rat liver mitochondria with trypsin. These results suggest that, in rat liver mitochondria, MAO B is situated on the surface of the MOM facing the intermembrane space, while MAO A is situated facing the cytosolic face of the MOM. Thus, the protease inactivation results are in agreement with the conclusions reached from inhibition studies with the TEMPO-pargyline analogues described above.

■ DISCUSSION

Comparison of TEMPO-Pargyline Reactivities of Rat and Human MAO A and MAO B. The absolute specificity of ParSL-2 for MAO B and ParSL-3 for MAO A is less strict with the rat enzymes than with the human recombinant enzymes. Differences in inhibitor specificities and reactivities between the rat and human enzymes have been observed previously,[15,16] and this study provides an additional example. The differential reactivities observed previously with intact mitochondria isolated from yeast containing recombinant human enzymes and from human placenta[14] are also observed in this study using recombinant rat enzymes in the yeast organelles and in rat liver. Thus, the membrane-bound human and rat enzymes exhibit similar sensitivities, reflecting their topological orientation.

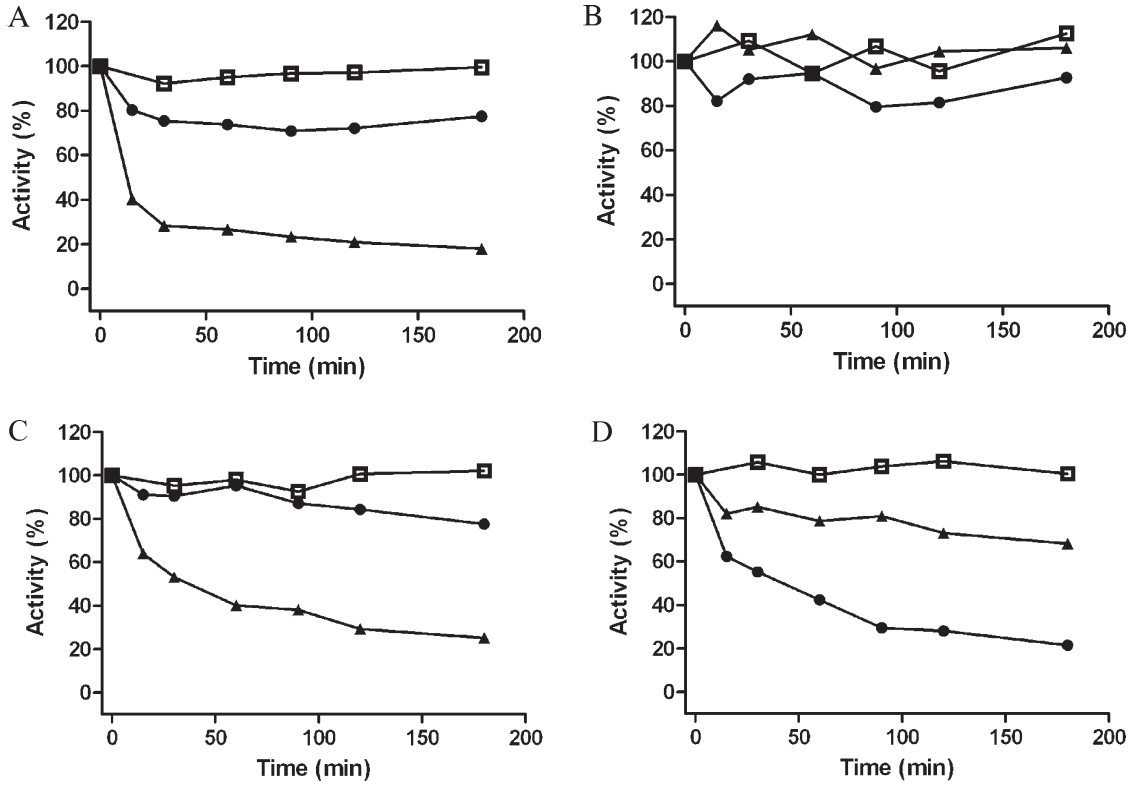

Figure 3. Comparison of the time dependence of rat MAO A and MAO B inhibition by ParSL-2 and ParSL-3 in intact and broken rat liver mitochondria, respectively: (A) intact mitochondria with serotonin as the substrate, (B) intact mitochondria with 3-AmMePy as the substrate, (C) broken mitochondria with serotonin as the substrate, and (D) broken mitochondria with 3-AmMePy as the substrate. Data for intact and broken mitochondria (not treated with ParSL-2 or ParSL-3) are shown (□). Data for mitochondria (intact or broken) treated with 1 mM ParSL-2 and 1 mM ParSL-3 are shown (● and ▲, respectively). The specific activities of rat liver mitochondria are 0.44 milliunits/mg (serotonin as the substrate) and 1.56 milliunits/mg (3-AmMePy as the substrate).

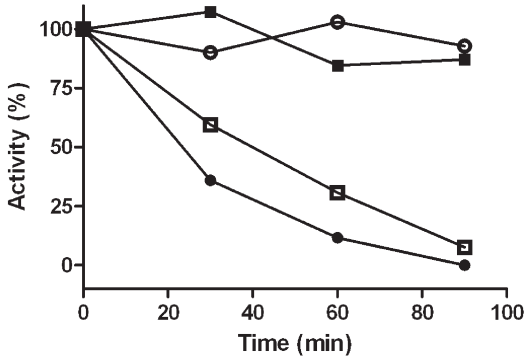

Figure 4. Comparison of activity losses on proteolytic treatment of yeast recombinant MOM-bound rat MAO A and MAO B: trypsin-treated rat MAO A (●), β-chymotrypsin-treated rat MAO A (○), trypsin-treated rat MAO B (■), and β-chymotrypsin-treated rat MAO B (□).

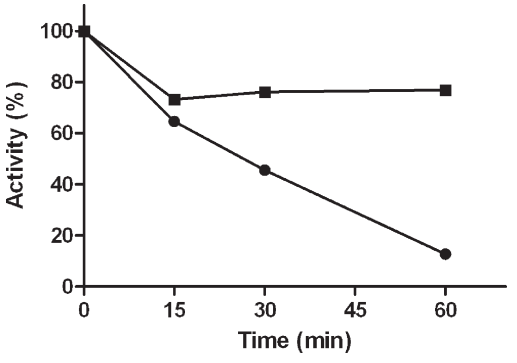

Figure 5. Comparison of activity losses on protease treatment of rat liver mitochondria: treated with trypsin using serotonin as the substrate (●) and treated with β-chymotrypsin using 3-AmMePy as the substrate (■).

Topology of Rat MAOs in Mitochondria from Different Sources. The mitochondrial outer membrane impermeability due to the polar nature of the amide-linked TEMPO group on these pargyline analogues has been suggested previously[14] and is also evident in this study with new insights into liver MAO B topology. The demonstrated reactivity of both MAO A and MAO B in intact mitochondria to pargyline but differential reactivity to TEMPO-pargyline analogues provides additional support for the inability of these analogues to traverse the mitochondrial outer membrane. The rat liver data support previous studies that showed that MAO A is situated facing the cytosolic face of the MOM as found with intact human placental mitochondria. These results agree with the protease studies of Buckman et al.[11] that showed that the serotonin oxidizing activity of rat liver mitochondria was sensitive to trypsin while the phenylethylamine oxidizing activity was relatively less sensitive. Our trpysin inactivation studies confirm the results of Buckman et al.[11] in that MAO A in intact rat liver mitochondria is situated on the cytosolic face. The recombinant rat enzyme in yeast mitochondria provides evidence of an opposite orientation in yeast (i.e., MAO A facing the intermembrane face).

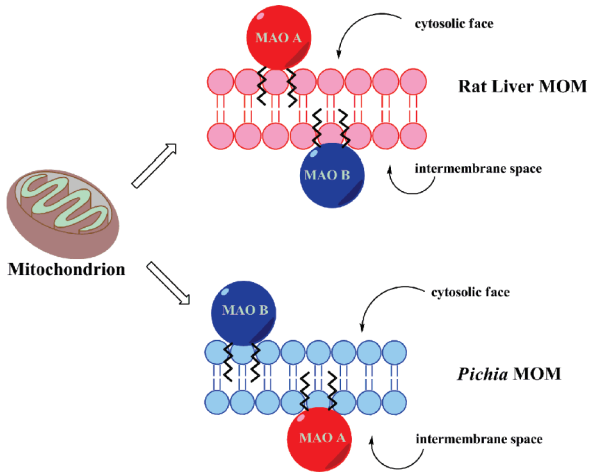

Figure 6. Depiction of topological orientations of rat MAO A and rat MAO B in rat liver MOM and in *P. pastoris* MOM.

An unexpected outcome of these studies is the demonstration that rat MAO B is situated facing the intermembrane face of rat liver mitochondria but the cytosolic face of yeast mitochondria. Support for this conclusion comes from the differential sensitivities of rat MAO B to inactivation in intact mitochondria isolated from rat liver and from the yeast expression system. These conclusions are presented as a cartoon in Figure 6.

The apparent opposite orientations found in *Pichia* mitochondrial preparations suggest that the lipid composition of yeast favors a differential orientation and therefore brings up a number of unanswered questions about how MAO A and MAO B are inserted in the MOM. Both enzymes have transmembrane C-terminal helices that are involved in membrane binding. Recent reviews[13,24] suggest this post-translational insertion can occur via a "spontaneous" insertion that may or may not require chaperone assistance. If this type of insertion is operative, then MAO B may elicit an auxiliary mechanism in rat liver to promote its translocation to the intermembrane face of the MOM.

The finding of opposite orientations of MAO in rat liver also brings up the question of whether such a topological arrangement is tissue-specific or is general for mitochondria isolated from differing tissues. This remains an open question requiring further experimental investigation. This situation is also relevant to the design of MAO B inhibitors as neuroprotectants. Functional MAO B inhibitors may have to be readily transported into the intermembrane space in mitochondria assuming a generality of topology. If there is a tissue specificity to topology, then an additional aspect of tissue specificity will have to be factored into MAO B-specific drug design.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Structures of the TEMPO-substituted pargyline analogues used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Biochemistry, Rollins Research Bldg., Emory University, 1510 Clifton Rd., Atlanta, GA 30322. E-mail: deedmon@emory.edu. Phone: (404) 727-5972. Fax: (404) 727-2738.

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■ ABBREVIATIONS

MAO, monoamine oxidase; MOM, mitochondrial outer membrane; TEMPO, 2,2,6,6-tetramethylpiperidinyl-1-oxyl; ParSL, spin-labeled pargyline; 3-AmMePy, 3-(2-aminomethyl)pyridine.

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