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Comparative membrane locations and activities of human monoamine oxidases expressed in yeast

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Human monoamine oxidases A and B were expressed under the control of a galactose inducible promoter in *Saccharomyces cerevisiae*. The two MAO isoenzymes were found located in the yeast mitochrondrial outer membrane, probably in different orientations as suggested by controlled proteolysis experiments. A high level of both human MAO-A or -B activities is measured in intact mitochrondria without the need for any detergent solubilisation step. The substrate and inhibitor selectivities of the membrane-bound MAOs are highly similar to those of purified human enzymes. The level of MAO-B activity, however, is selectively lowered when bound to the membrane.

Monoamine oxidase; Yeast expression; Polymerase chain reaction; Mitochrondrion; Human; *Saccharomyces cerevisiae*

1. INTRODUCTION

Monoamine oxidases (MAO, EC 1.4.3.4) are higher eucaryote integral proteins of the outer mitochondrial membrane. These flavoenzymes catalyze the oxidative deaminations:

$$R\text{-}CH_2NH_3^+ + O_2 + H_2O \longrightarrow R\text{-}CHO + H_2O_2 + NH_4^+$$

of several exogenous and endogenous amines in various tissues [1]. Two isoenzymes, encoded by separate genes [2,3], and having different substrate preferences and inhibitor specificities are responsible for human MAO activity. MAO-A oxidizes vasoactive and neuroactive amines, such as serotonin, and is preferentially inactivated by the acetylenic inhibitor clorgyline. MAO-B metabolizes xenobiotic amines and is preferentially inactivated by pargyline [4–6].

In almost all human tissues, MAO activity consists of a mixture of both forms of the enzyme; although placental tissue contains predominantly MAO-A and platelets mainly MAO-B [1,7]. The difficulty in obtaining large amounts of a pure MAO form from human sources and the low expression level in transfected mammalian cells [8], makes heterologous expression in yeast a tempting alternative. Very recently, the human A-isoenzyme was expressed and purified from transformed yeast cells, but no data were provided concerning its subcellular location and functionality in the yeast membranes [9]. We extend the study to the B-isoenzyme and investigate the MAO membrane locations and functionalities in yeast cells. The effects of the membrane environment on MAO activities are also analyzed since the lipid composition was described to have an influence [1,10]. Part of this work has been presented before in preliminary form [11].

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2. MATERIALS AND METHODS

Chemicals were purchased from Sigma. *Thermus aquaticus* DNA polymerase was from Bioprobe Systems. Oligonucleotides were synthesized on a MilliGen 7500 DNA synthesizer and purified by DEAE-Sephacel (Pharmacia) chromatography and repeated ethanolic precipitations. PCR amplifications were performed using standard conditions described previously [12]. The *Saccharomyces cerevisiae* W303.1B strain (*MAT*a, *leu*2, *his*3, *trp*1, *ura*3, *ade*2-1, can^R, cyr$^+$) was provided by Dr P. Netter. The expression plasmid pYeDP1/8-2 was described previously [13]. The human cDNA encoding MAO-A was obtained as pHM11 plasmid [3]. The DNA fragment encompassing the human MAO-B coding sequence was obtained by using PCR amplification from adult human liver total mRNAs. This DNA fragment was cloned into the *Xba*I-*Pst*I sites of Bluescript II-KS vector (Stratagene) yielding the BSMAOB plasmid. Sequencing of the PCR-amplified material revealed that only 2 base pairs (bp) differ from the published sequence [2], each in the 3'-non-coding region.

Culture media SWA5 (containing galactose) and SWA6 (containing glucose), spheroplast preparation by enzymatic digestion of yeast cell walls, and subcellular fractionations were described previously [13,14]. A crude mitochondrial fraction resulting from about 4×10^9 cells was resuspended in 2 ml of 50 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 0.6 M sorbitol and 25% double-distilled glycerol, and centrifuged for 3 min at 3000 rpm in a microcentrifuge, the pellet was discarded and the supernatant centrifuged again for 6 min at 10000 rpm. The mitochondrial pellet was resuspended and the same operation repeated twice. The final purified mitochondrial pellet was resuspended in the same buffer at 3–5 mg protein/ml, and kept frozen at −70°C for weeks without any detectable change in MAO activity.

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Serotonin deaminase activity was assayed in a 1-ml assay mixture of 50 mM phosphate buffer pH 7.5 containing 0.1 mM serotonin, and the mitochondrial aliquot (10 μg of protein). After a 10-min incubation at 37°C, the reaction was quenched by the addition of 0.1 ml 6 M HCl. The reaction product was extracted with 1 ml of ethylacetate/toluene (1 : 1, vol/vol), and quantified by its fluorescence emission spectrum between 320 and 400 nm (excitation wavelength 289 nm). Kynuramine deaminase activity (MAO-A) and benzylamine deaminase activity (MAO-B) were measured as described [15] using 15–30 μg of protein. Protein concentrations were determined by the Pierce BCA protein microassay using bovine serum albumin as a standard.

The digitonin solubilization of mitochondrial outer membrane proteins was carried out by mixing the sample (0.3 mg proteins) with an equal volume of 2% digitonin in 50 mM Tris-HCl buffer pH 7.4, 1 mM EDTA, and then incubating the mixture for 10 min at 37°C. The suspension was then centrifuged for 10 min at 10000 rpm. The supernatant contains the digitonin-solubilized proteins, while the pellet contains membranes. Clorgyline and pargyline titrations of mitochondrial aliquots were performed by incubating 20–40 μg proteins with various concentrations of acetylenic inhibitor for 15 min at 37°C. 100% relative activity corresponds respectively to 42 and 5 nmol of substrate/min/mg proteins for MAO-A and MAO-B assays.

Controlled proteolysis using Proteinase K (Boehringer) on yeast mitochondria (0.2 mg proteins) was carried out in 0.25 ml of isoosmotic buffer, Tris-HCl 50 mM pH 7.4, 1 mM EDTA, 0.6 M sorbitol, 25% glycerol. Incubations with 60 μg or 200 μg Proteinase K per mg of mitochondrial protein were at 37°C for 45 min. Control experiments were run in parallel without Proteinase K. MAO activities were normalized using cytochrome c oxidase activity [16] as a reference.

3. RESULTS AND DISCUSSION

3.1. *Construction of yeast strains expressing human MAO-A and -B*

Heterologous expression in yeast cells requires the reformatting of the heterologous cDNA by removing all the 5′-non-coding sequence prior to insertion into the yeast expression vector. This last step maximizes the level of expression, as previously shown [13,14]. The *Bst*UI-*Eco*RI 1880 bp fragment of pHM11 [3] that includes the MAO-A coding sequence minus the first 28 nucleotides, and 300 bp of the 3′-non-coding sequence, was ligated with the synthetic oligonucleotide: ATGGAAAACCAAGAAAAGGCTTCTATCG in order to restore the full length coding sequence. Nucleotides modified so as to match the yeast codon bias are underlined. These substitutions do not change the deduced amino acid sequence. The resulting 1910 bp fragment formatted as a *Bgl*II-*Eco*RI cassette was inserted between the *Bam*HI and *Eco*RI sites of pYeDP1/8-2 expression vector, to give pHMAOAV8. PCR amplification using primers deduced from the published sequence [2] was used to extract the MAO-B cDNA coding sequence from pBSMAOB. This procedure introduces a *Bgl*II site immediately upstream of the start codon ATG and a second *Bgl*II site overlapping partially TAA stop codon at the 3′-end. The resulting *Bgl*II cassette was inserted into the *Bam*HI site of pYeDP1/8-2 expression vector in the proper orientation yielding pHMAOBV8 (see Fig. 1).

3.2. *Subcellular location of HMAO-A and -B expressed in yeast cells*

W303.1B yeast cells transformed by plasmids HMAOAV8 and HMAOBV8 were selected for growth on uracil-depleted minimal medium containing glucose. The expression of the heterologous MAO genes was

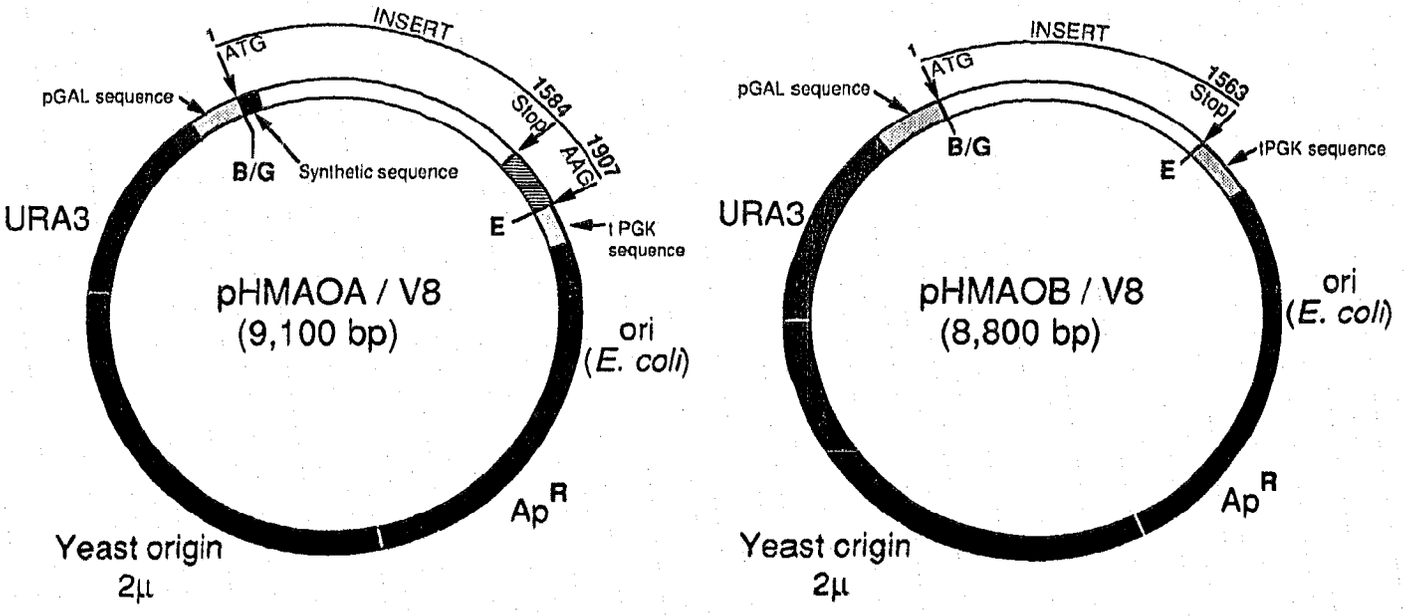


Fig. 1. Constructs for expression of human MAOs in yeast. In the MAO-A expression construct, the 1907 bp insert is composed of the 28 bp synthetic 5′-coding sequence (black box), the 1556 bp remaining coding sequence, and a 3′-non-coding sequence (hatched box). In the MAO-B expression construct, the insert is composed of the 1560 bp coding sequence including the stop codon. B, G and E, respectively stand for *Bam*HI, *Bgl*II and *Eco*RI.

Table I

Subcellular locations of yeast-expressed human MAOs

Substrate	Yeast	Activity in		
		Mitochondria	Microsomes	Cytosol
Serotonin	Expressing MAO-A	50.0 (45000)	0.5 (5000)	0.1 (1000)
	Control	≤0.01	≤0.01	≤0.01
Benzylamine	Expressing MAO-B	6.1 (14000)	0.3 (3000)	0.15 (2500)
	Control	≤0.06	≤0.03	≤0.03

Specific activities are in nmol/min/mg protein. Values between brackets are total MAO activities (in pmol/min/4 × 10^9 cells).

started by replication on galactose-containing plates, followed by well-aerated culture in SWA5 liquid medium. Cell lysis was performed under mild and iso-osmotic conditions, after digestion of the cell wall by a mixture of lytic enzymes. During the purification procedure, particular caution was taken to avoid physical damage to mitochondria (see section 2). The different subcellular fractions were isolated and examined for the typical serotonin and benzylamine deaminase activities of human MAO-A and -B, respectively. Table I indicates that a high level of the corresponding MAO activities is found in yeast mitochondrial fractions when cells are transformed to express either A- or B-isoenzyme. On the contrary, mitochondria from control yeast did not exhibit any detectable MAO activity when tested with the same substrates. On the basis of the turn-over numbers determined on purified human placental MAO-A or bovine liver MAO-B [15], a mitochondrial content of, respectively, 520 and 25 pmol per mg protein can be estimated. The total mitochondrial activity represents 88% of the total cellular activity for MAO-A and 72% of that for MAO-B. We thus conclude that both MAO isoenzymes are functionally expressed and localized mainly in yeast mitochondria. This situation is highly reminiscent of the human case [17].

In order to test the submitochondrial location(s) of MAOs and the influence on their activities of the membrane environment, we carried out the disruption of mitochondrial outer membrane by digitonin [18]. Such a treatment results in the apparent solubilization of most if not all the deaminase activities (Table II), indicating an outer membrane location in yeast for the two human isoenzymes. Surprisingly enough, a 3-fold increase in the B, but not A, activity was observed upon digitonin solubilization. This observation may be related to the known inhibitory effect selective for MAO-B of phosphatidylserine [19], and points to the fact that the mitochondrial membrane containing MAO-B is a more physiological model than the purified enzyme.

To further characterize MAO membrane location(s), intact mitochondria were submitted to controlled proteinase K digestions under iso-osmotic conditions. Residual activities were standardized in order to correct them for artifacts due to damaged mitochondria by using cytochrome *c* oxidase activity as a reference. Fig. 2 shows that both MAO-A and -B are partially sensitive to limited proteolysis suggesting an outer side location. Moreover, a differential behaviour between MAOs was observed when similar analyses were performed at two concentrations of proteinase K. The results suggest that if both MAOs partition between the two sides of the outer membrane, MAO-B seems mainly located on the outer face. However, the slightly different behaviours between the MAOs observed in this study, made in the same membrane context (yeast mitochondria), are consistent with the previous hypothesis concerning the relative orientation of MAO-A in human mitochondria from brain cortex (synaptosomal and non-

Table II

Digitonin treatment of MAO-expressing yeast mitochondria

Protein expressed	Substrate	Total activity in pmol/min		
		Before treatment	After treatment	
			Pellet	Supernatant
MAO-A	Kynuramine	730	0	690
MAO-B	Benzylamine	740	50	2290

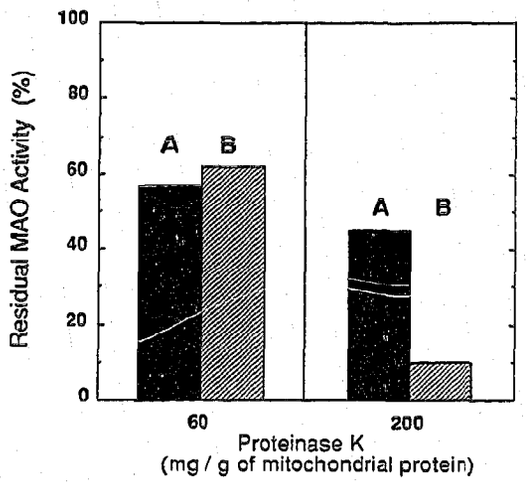
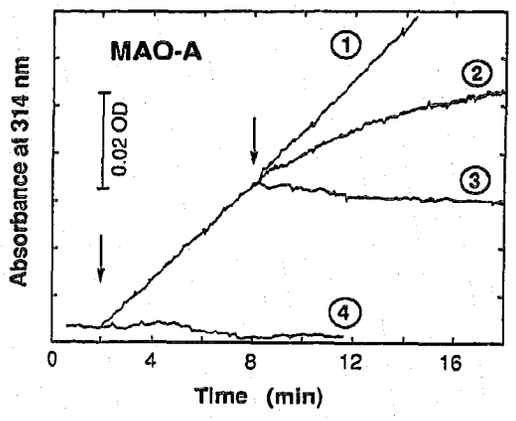


Fig. 2. Controlled proteolysis in iso-osmotic conditions on yeast mitochondria expressing MAOs. A refers to the A-isoenzyme, B to the B isoenzyme.

synaptosomal), and of MAO-B from liver [20]. The mitochondrial addressing of MAOs in yeast thus seems to be similar to that in the human.

3.3. *Substrate and inhibitor specificities of membrane-bound MAOs*

Most of the MAO substrates and inhibitors are relatively hydrophobic and accumulate preferentially in the membrane phase. Moreover, detergent solubilization of MAOs for purification purposes was reported to affect their kinetic properties [21]. Therefore, we questioned the possible role of the membrane on human MAO activities expressed in yeast. The mitochondrial fractions containing MAOs were tested for typical serotonin (MAO-A) and benzylamine (MAO-B) activities without a solubilization step and in the same cellular context (yeast cells), contrary to usual kinetic studies made with the purified form from either human placenta (MAO-A) or bovine liver (MAO-B). In Table III the obtained steady-state parameters are compared with data reported for MAO purified from human tissues [4,22], or expressed in transfected COS cells [8]. For both MAOs the measured K_m values are similar,



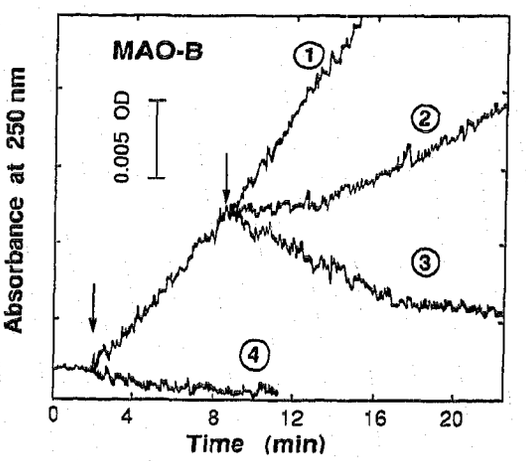


Fig. 3. Time courses of kynuramine (MAO-A) and benzylamine (MAO-B) deaminations using yeast mitochondria. For both panels, the 2 min arrow indicates the addition of the mitochondrial aliquot: traces 1 to 3, 20 μg (MAO-A) or 50 μg (MAO-B) of protein; trace 4 (control yeast) 60 μg proteins. Additions at 8 min were: trace 1, buffer (MAO-A and-B); trace 2, pargyline final conc. 94 μM (MAO-A) or clorgyline final conc. 10 μM (MAO-B); trace 3, clorgyline final conc. 9 μM (MAO-A) or pargyline final conc. 19 μM (MAO-B).

whatever the system considered. In contrast, V_m values per mg of protein are at least 10-fold higher in yeast mitochondria than in the other systems. This can be interpreted either as a higher expression level or as an im-

Table III

Steady-state parameters determined with yeast mitochondrial fraction

Enzyme		Source of mitochondria		
		Yeast[a]	Human tissue	COS cells[b]
MAO-A (serotonin)	K_m (in μM)	100 ± 10	95[c]	–
	V_{max}[d,e]	50 ± 3	0.8[b]	0.2
MAO-B (benzylamine)	K_m (in μM)	60 ± 10	64[c]	–
	V_{max}[d]	6.4 ± 0.6[f]	0.6[b,e,g]	0.2[e,g]

Comparison with human MAOs from other sources. a, this work; b, ref. [8]; c, ref. [22]; d, V_{max} value is expressed in nmol/min/mg protein; e, incubations at 37°C; f, incubations at 20°C; g, phenethylamine as substrate.

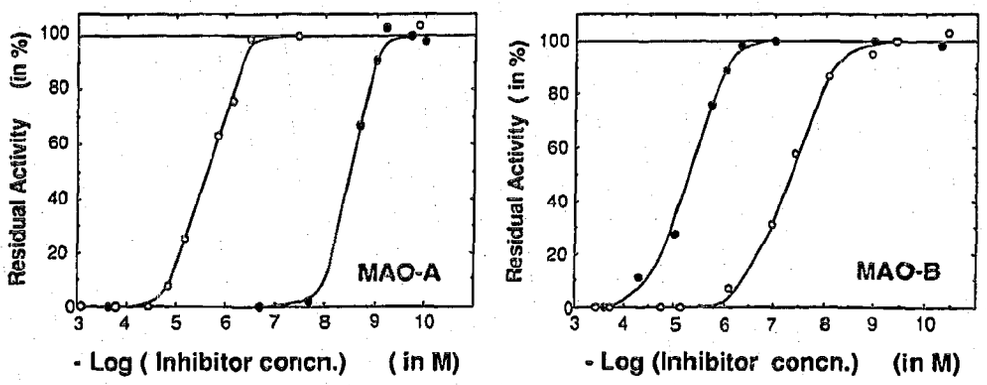


Fig. 4. Effects of varying clorgyline (•) and pargyline (○) concentration on MAO activity expressed in yeast mitochondria.

proved turn-over number. To further investigate the possible membrane environment effects, we analyzed the effect of two isoenzyme-specific inhibitors: clorgyline (MAO-A) and pargyline (MAO-B). Addition of 9 μM clorgyline to mitochondria expressing MAO-A resulted in a complete and immediate loss of the kynuramine deaminase activity (Fig. 3, panel MAO-A trace 3), whereas 94 μM pargyline inactivated expressed MAO-A in a slow process (trace 2). Addition of 19 μM pargyline to mitochondria expressing MAO-B resulted in a complete and immediate inactivation of the heterologous enzyme (panel MAO-B, trace 3). The transient absorbance decrease occurring immediately after pargyline addition is likely related to some changes in the diffusion level of the mitochondria suspension. A similar negative slope is occasionally observed in control experiments. Whereas addition of 10 μM clorgyline did not result in inactivation of MAO-B, but caused a typical competitive inhibition (trace 2). To extend the preceding data, we carried out the titration studies shown in Fig. 4. Comparison with similar experiments done on enzymes purified from mammalian tissues [23,24] indicates that the inhibitor selectivity of MAOs in yeast mitochondrial membranes is highly similar to the responses observed with authentic purified MAOs. Therefore, yeast mitochondria containing human MAOs appear to be reliable model systems simulating the natural membrane environment, a point of importance when the effects on MAO activity of the membrane context are taken in account. Moreover, this system is more convenient than purified enzymes since it permits one to produce reproducible material in large amounts. Such a model can be of potential importance in facilitating the development of anti-MAO drugs.

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