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Lipid–Protein Interactions in the Multiple Forms of Monoamine Oxidase

Enzymatic and ESR Studies with Purified Intact Rat Brain Mitochondria

ROSA H. HUANG

Department of Biochemistry, College of Medicine, University of South Alabama, Mobile, Alabama 36688

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SUMMARY

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The temperature dependence of the initial velocity, v_0, using substrates benzylamine, tryptamine, tyramine, and serotonin for the multiple enzyme forms of monoamine oxidase *in situ* in purified intact rat brain mitochondria, was determined in the range between 4° and 45°. In rat brain tissue, the oxidative deamination of serotonin is catalyzed by monoamine oxidase-A-type enzyme exclusively. In contrast, the oxidation of benzylamine is catalyzed by monoamine oxidase-B-type enzyme. However, both enzyme types metabolize tryptamine and tyramine. Arrhenius plots reflecting the functional states of the multiple monoamine oxidase enzyme forms showed clear differences. A straight line was observed when using benzylamine as substrate. Changes in slope at 21–22° and 35–36° were observed when serotonin, tryptamine or tyramine was employed as substrate. The functional state of monoamine oxidase-B enzyme appeared to be homogeneous whereas that of monoamine oxidase-A enzyme appeared to be heterogeneous and strongly temperature dependent. Spin-labeled stearic acids I (12, 3) or I (1, 14) were incorporated as structural probes into the outer mitochondrial membrane. Temperature dependence of order parameter S, indicating fluidity in the vicinity close to the surface region of the membrane, revealed apparent breaks at 21–22° and 32.6–33.6° when I (12, 3) was used. Temperature dependence of the rotational correlation time τ, relating to fluidity in the center region of the membrane, indicated phase transition at 29–30° when I (1, 14) was used. The functional state of monoamine oxidase-B enzyme was apparently independent of the fluidity properties of the membrane lipids. Coincidence of transition temperature at 21–22° was observed only when spin-labeled stearic acid I (12, 3) was used in the ESR study and ^{14}C-labeled substrates serotonin, tyramine or tryptamine was used in the enzymic study. The nature of monoamine oxidase-A enzyme was sensitive to the physical state of the bulk membrane lipids through lipid–protein interactions. The lipid–protein interactions occurring in the region close to the surface of the membrane were effective in modulating the functional state of monoamine oxidase-A enzyme. The break temperature at 35–36° observed using tryptamine, tyramine, or serotonin as substrate for the monoamine oxidase-A enzyme—did not correspond to any of the characteristic phase transition temperatures of the bulk membrane-lipid domain. A temperature-induced conformational change in monoamine oxidase-A at 35–36° may be attributed to an additional lipid–protein interaction originating from the tightly bound lipid shell or an intrinsic alteration in the enzyme protein.

INTRODUCTION

The oxidative deamination of biogenic monoamines is now believed to be accomplished by at least two principal, functionally different forms of monoamine oxidase[1] (MAO, EC 1.4.3.4) (1, 2). In rat brain tissue, MAO-A-type enzyme which deaminates serotonin is sensitive to clorgyline inhibition. MAO-B-type enzyme which preferentially deaminates benzylamine is sensitive to de-

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[1] The abbreviation used is: MAO, monoamine oxidase (EC 1.4.3.4).

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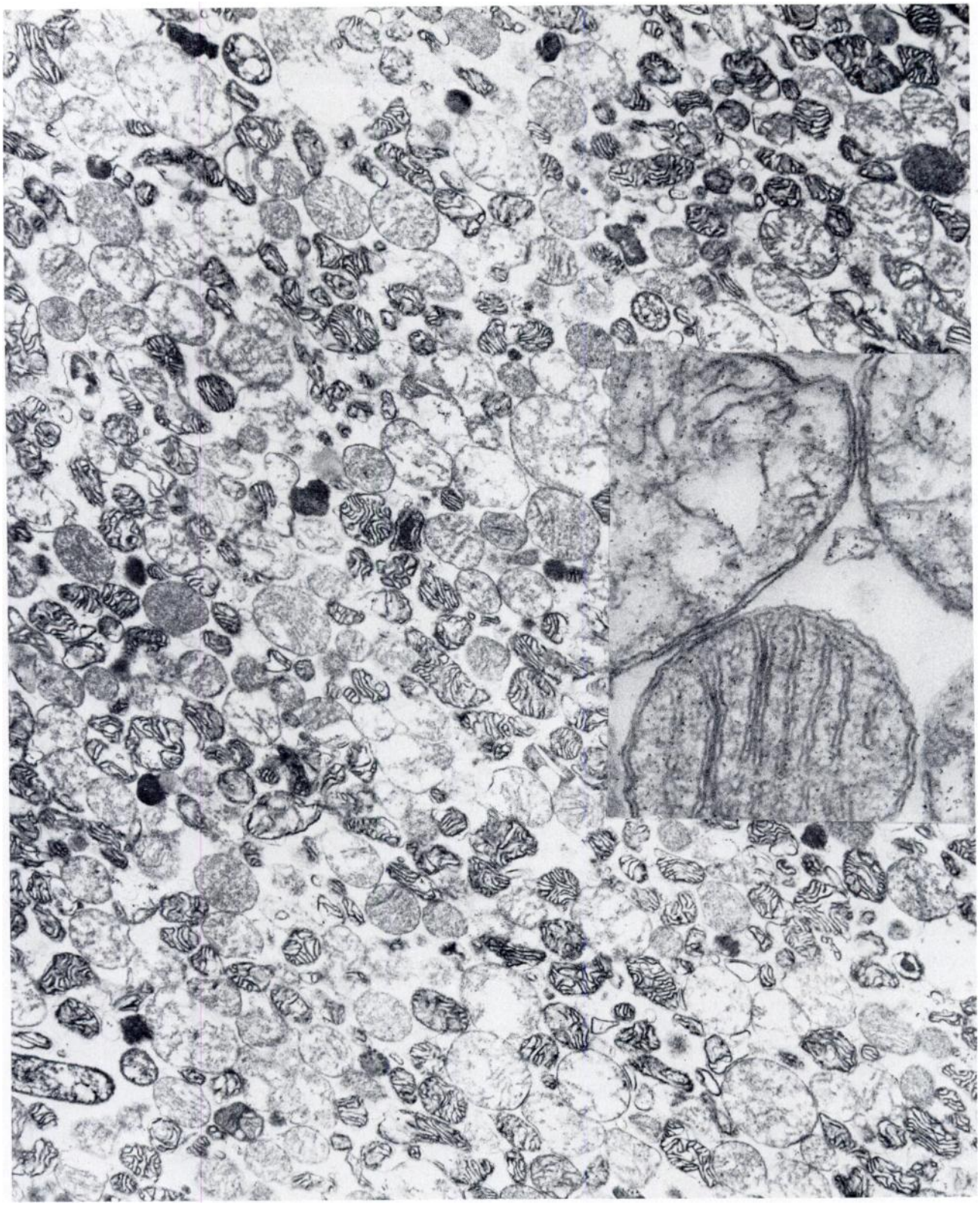


FIG. 1. *Electron micrograph of pure intact rat brain mitochondrial preparation, magnification 4500×*
The inset illustrates the intact double-membrane feature of rat brain mitochondria after the spin-labeling treatment, magnification 34,000×.

prenyl inhibition. Tyramine and tryptamine are substrates for both MAO-A and MAO-B enzymes.

Within the cell, MAO, an intrinsic membrane-bound flavoprotein, has been localized in the outer mitochondrial membrane (3, 4). Procedures involving prolonged sonication and/or detergent treatment are required to

solubilize the enzyme (5). Apparent K_m and substrate–inhibitor sensitivities are altered as a consequence of solubilization (6–8). A number of discrete MAO-active fractions or bands obtained by column chromatography, isoelectric focusing or polyacrylamide gel electrophoresis of solubilized preparation failed to relate to the two principal functional MAO forms unambiguously (7, 9). It was suggested that the observed multiple fractions of monoamine oxidase may be an artifact of purification procedures. However, treatment of the MAO-active bands with chaotropic agents resulted in the disappearance of the apparent multiplicity of the enzyme both in terms of electrophoretic mobility and substrate–inhibitor specificity (1, 10). MAO-A and MAO-B may be the same enzyme protein in different lipid environment. In addition, the lipophilicity of pargyline analog was closely correlated to its inhibitory potency (11). More recently, using spin-label probe (spin-labeled hydroxyamphetamine, a competitive reversible inhibitor) it was demonstrated that these multiple forms of monoamine oxidases did exist *in situ* in the membrane of intact mitochondria and their substrate–inhibitor specificities were strongly suggested to depend upon the properties of membrane lipids through intimate lipid–protein interactions (8).

The aim of the present work has been to further study the nature of lipid–protein interactions specific to MAO-A or MAO-B by investigating the interrelationship of the physical state of the lipid-bilayer domain and the functional state of the membrane-bound MAO-A and MAO-B enzymes. To this purpose, independent but parallel studies of the temperature dependence of monoamine oxidase activity using substrates specific to MAO-A and/or MAO-B enzymes and the membrane fluidity using stearic acids were undertaken. Spin-labeled stearic acids were incorporated into the outer membrane of intact brain mitochondria preparation. The electron paramagnetic resonance spectra of such spin-labels are well understood from the investigation of model systems. Information concerning the motion of the spin label in its particular environment may be deduced by these methods (12–15).

MATERIALS AND METHODS

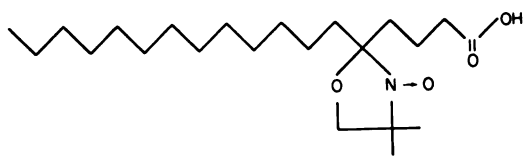
Chemicals. The chemicals used in this work were obtained as follows: spin-labeled stearic acids I (12, 3) [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl] and I (1, 14) [2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl] from SYVA Corporation, Palo Alto, California; [*methylene*-^{14}C]benzylamine hydrochloride from International Chemical and Nuclear Corporation; [1-^{14}C]tyramine hydrochloride, β-[^{14}C]-tryptamine bisuccinate, β5-hydroxy[^{14}C]tryptamine bioxalate from New England Nuclear. Other chemicals were obtained from commercial sources.

Pure intact rat brain mitochondria were prepared by purifying crude mitochondrial fractions from rat brain (male, Sprague–Dawley, weight 120 to 180 g) utilizing a discontinuous Ficoll density gradient (3 and 6%) procedure (16). The preparation determined by electron microscopy to be intact and contained at least 90% mitochondria with minute synaptosomal contamination (Fig. 1).

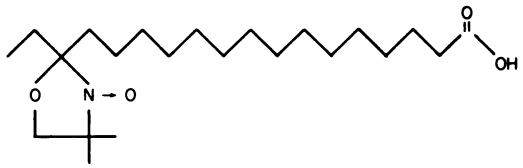
Protein concentrations used in the calculations of specific activities were measured by the method of Lowry *et al.* (17), using bovine serum albumin as standard.

Measurement of monoamine oxidase activity. Four different substrates were used to determine monoamine oxidase activity. The isolated intact rat brain mitochondria obtained from two rat brains were suspended in a final volume of 2.5 ml at 0° containing 0.25 M sucrose, 10 mM Tris, 0.5 mM Na^+-EDTA at pH 7.4. The protein concentration of the suspension varied from 2 to 3 mg/ml. The monoamine oxidase activity was determined in duplicate by incubating 0.20 ml aliquots of the mitochondrial suspension in 1 ml of 0.05 M potassium phosphate buffer, pH 7.4, with ^{14}C-labeled substrates for 30 min at incubating temperatures ranging from 4° to 45° with increments of approximately 2°. The final concentration for all the substrates used for measurements at all temperatures was 2 mM. The selected substrate concentration was determined based on two criteria; linearity of product formation with time at constant enzyme concentration; in addition, the product formation per unit time was also linear with the amount of enzyme at three different assaying temperatures, i.e., 10, 37 and 45°. Therefore, the specific activity obtained over the temperature range represented the initial velocity, v_0, which in turn, characterized the functional state of the multiple forms of monoamine oxidase. The method of Robinson *et al.* (18) was modified when serotonin and tyramine were used as substrates. After incubation, the reaction was stopped by rapid cooling in ice bath and the product was separated by passing the reaction mixture through an Amberlite column (0.5 × 4 cm). The product was collected and counted in 10 ml of Aquasol. The method of Wurtman and Axelrod was used when benzylamine and tryptamine were substrates (19). The reaction was terminated by adding 0.1 ml of 6 N HCl. Toluene, 6.0 ml, was then added and the product was extracted and counted in 10 ml of scintillation fluid 2,5-diphenyl-oxazole (PPO) 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), toluene.

ESR measurements. Spin labels we have used, *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of stearic acid I (12, 3) and I (1, 14), differ from each other in the position of the spin-label group with respect to the chain ends.



I (12, 3)



I (1, 14)

The spin labels were incorporated into the outer mitochondrial membrane by the following procedure. The spin labels were dissolved in ethanol at 2 mg/ml; 5–10

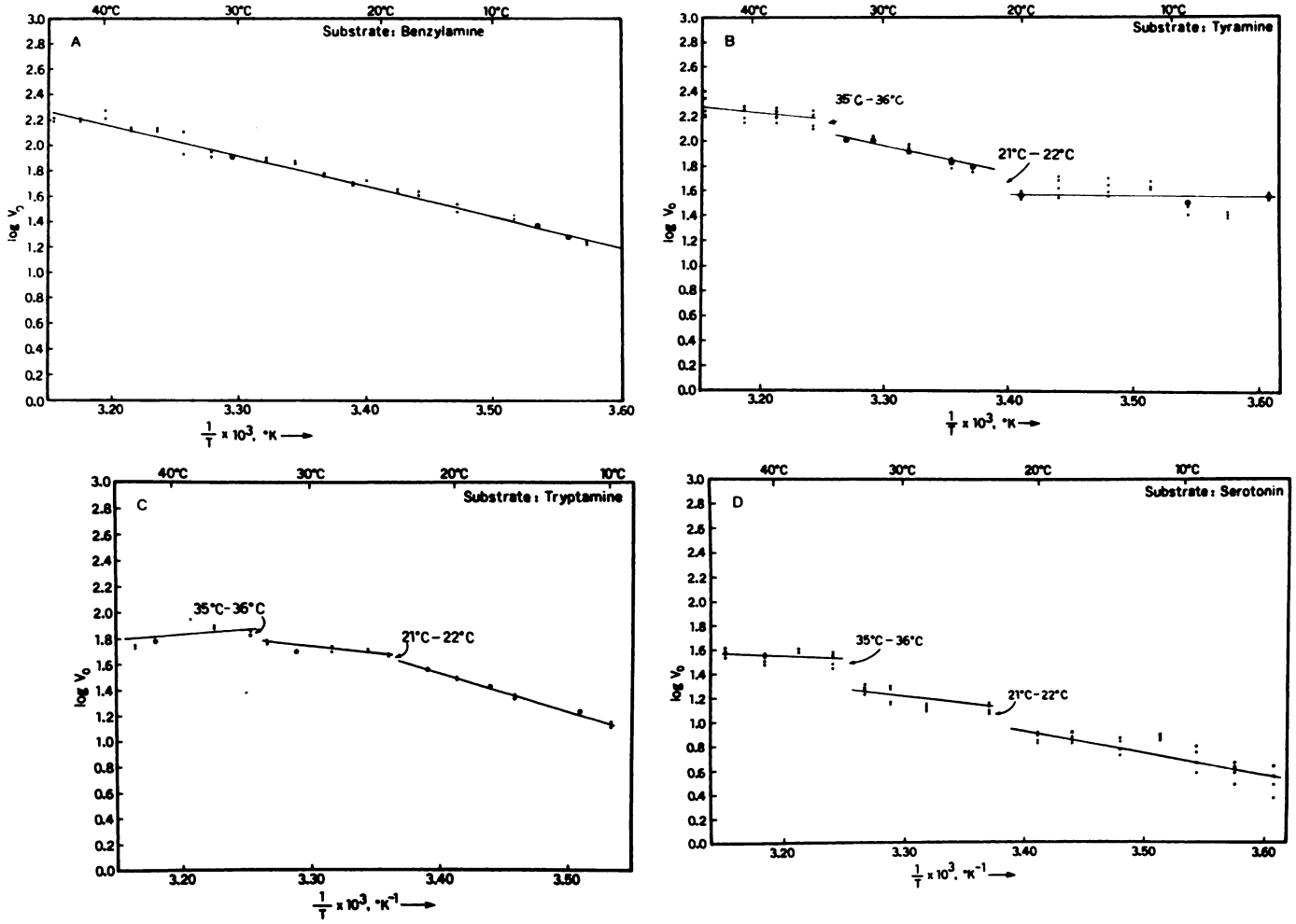


FIG. 2. *Arrhenius plots of log v_0 vs 1/T*

(A) Benzylamine as substrate, data from two independent experiments. (B) Tyramine as substrate, data from four independent experiments. (C) Tryptamine as substrate, data from two independent experiments. (D) Serotonin as substrate, data from four independent experiments. • represents one datum point which is the average of a duplicate determination for one independent experiment. ● represents two coinciding datum points.

μl of the ethanol solution of the spin label was pipetted into the bottom of a 10-ml test tube. The solvent was evaporated completely under a stream of dry N_2 while the tube was rotating, resulting in a thin dry film of spin label at the bottom portion of the test tube. Freshly prepared pure intact brain mitochondrial suspension (in isotonic medium) was added into the test tube. Then the sample was gently shaken on a Dubnoff shaking incubator at room temperature for less than 1 min. The outer mitochondrial membrane retained its integrity after the labeling treatment was demonstrated by electron microscopy (Fig. 1).[2] The spin labels were incorporated almost instantaneously into the outer membrane of the intact mitochondria. No change in the characteristics of spectrum was observed when spin-labeled mitochondria were washed extensively with isotonic medium. The label was not adventitiously adhering to the membrane. The concentration of the mitochondrial suspension was about 10 mg/ml. At maximum, the spin labels were added at a ratio of 0.75–1.5 mg spin label/100 mg of mitochondrial protein. Similar ratios have been used by other workers (20). The sample was transferred immediately to a variable-temperature aqueous solution sample cell. Spectra were recorded using Brucker ER 200tt EPR spectrometer over the temperature range 4–45° by increments of 2°. The temperature at the cell was regulated to be stable to about ±0.1°. The incubation time at each temperature prior to recording was 5 min.

[2] Our attempt of isolating the outer membranes from purified intact rat brain mitochondria by the large-amplitude swelling method described by Parsons *et al.* (29) indicated that the outer membrane was swollen but still attached and unbroken even after 1 hr incubation with swelling medium at 4°. Furthermore, a washing procedure using medium containing 0.5% Triton had minimal effect on brain mitochondria but solubilized 95% of the protein present in liver mitochondria.

RESULTS

Temperature dependence of the activities of the multiple functional forms of monoamine oxidase. When benzylamine was the substrate, the Arrhenius plot produced a straight line (Fig. 2A); since benzylamine is specific to the MAO-B form in rat brain mitochondria, this linear relationship suggested MAO-B enzyme was homogenous over the temperature range studied (4–45°).

Breaks occurred at 21–22° and 35–36° when serotonin, tyramine and tryptamine were the substrates for the enzyme (Fig. 2B–D).

The physical state of mitochondrial membrane as a function of temperature. A series of electron paramagnetic resonance spectra were obtained with intact brain mitochondria spin labeled with I (12, 3) for temperatures ranging from 4° to 48° with increments of 2°. Representative spectra at temperatures 8.5°, 25° and 44° may be seen in Fig. 3. These spectra are characteristic of an anisotropic rotation of the spin label around the long molecular axis. The order parameter S defines the mean angular deviation of the oscillations of the long molecular axis of I (12, 3). From the measured spectra, the order parameter S can be determined by the equations:

$$S = \frac{(T_\parallel - T_\perp')}{T_{zz} - \tfrac{1}{2}(T_{xx} + T_{yy})} \cdot \frac{a}{a'}, \qquad [1]$$

$$a = \tfrac{1}{3}(T_{zz} + T_{yy} + T_{xx}), \qquad [2]$$

$$a' = \tfrac{1}{3}(T_\parallel + 2T_\perp'), \qquad [3]$$

and

$$T_\perp' = T_\perp + 0.8\ G, \qquad [4]$$

where $T_\parallel$ and $T_\perp$ correspond to the separation of the outer and inner hyperfine extrema as shown in Fig. 3; T_{zz}, T_{xx} and T_{yy} are the principal elements of the T tensor (21).

Temperature dependence of the calculated order parameter S revealed apparent breaks at 21–22° and 32.6–33.6° (Fig. 4). The position of nitroxide spin label in I (12, 3) is close to the polar head group of the molecule. These breaks are interpreted as characteristic phase transition temperatures proximal to the surface region in bulk lipid-bilayer domain of outer mitochondrial membrane.

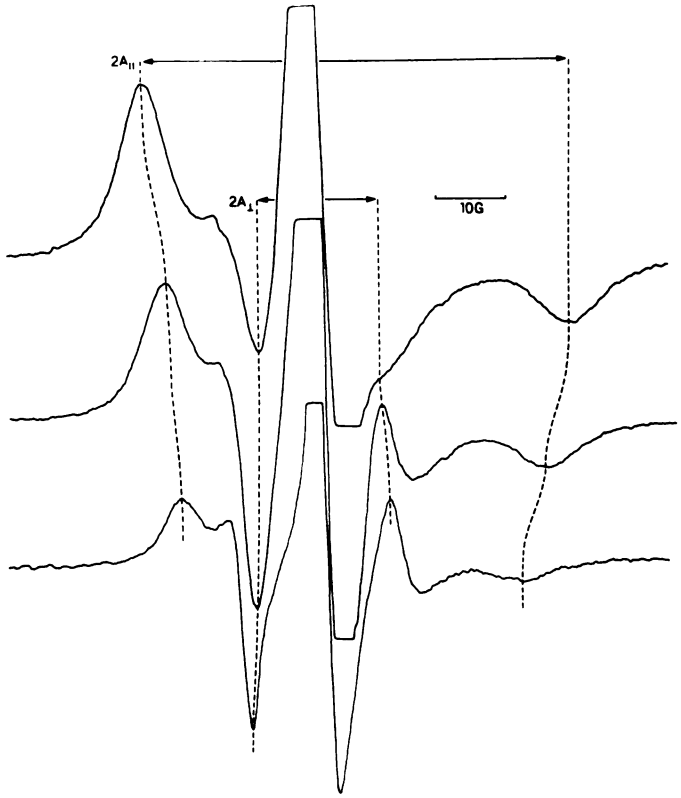


FIG. 3. *Spectra of I (12, 3) incorporated into the outer membranes of brain mitochondria at 8.5° (top), 25° (middle), and 44° (bottom)* $2A_\perp = T_\perp$, $2A_\parallel = T_\parallel$, $H_0 = 3490$ G.

Figure 5 shows ESR spectrum obtained with I (1, 14) which has the nitroxide spin label at a more distal position from the polar group. In this case, the physical properties of the center region of bulk membrane lipids were probed. The spectrum indicates relatively fast and isotropic motion of the spin labels. The correlation time, τ, reflecting the microviscosity of the environment can be extracted from the measured spectra by the formula in this motional tumbling range (13, 22).

$$\tau = 6.5 \times 10^{-10}\ \Delta H_0\ [(h_0/h_{-1}) - 1]^{1/2}, \qquad [5]$$

where ΔH_0 is the width of central line, h_0 and h_{-1} are the peak-to-peak heights of the mid- and high-field lines of the first derivative absorption spectrum. A semilogarithmic plot of τ versus $1/T$ showed a break at 29–30° (Fig. 6).

DISCUSSION

The functional state of the membrane-bound MAO-A and MAO-B was ascertained by studying the temperature dependence of the initial velocities v_0 of the enzymes using substrates specific to MAO-A and/or MAO-B enzymes. Arrhenius plot displayed a straight line when using benzylamine as a MAO-B specific substrate (Fig. 2A). The functional state of MAO-B appeared to be homogeneous. In contrast, three intersecting straight lines were observed when using serotonin, tryptamine or tyramine as the substrate for the enzymes (Figs. 2B–D). The observed temperature-dependent transitions in v_0 at 21–22° and 35–36° were attributed to the heterogeneous functional states of MAO-A enzyme, which was solely responsible for the oxidative deamination of serotonin. The distinctly different temperature dependence of MAO-A and MAO-B support and reinforce the hypothesis that at least two independent kinds of substrate binding sites coexist *in situ* in the membrane as suggested by our laboratory and other investigators (7, 8, 23, 24). It has also been reported that the activity associated with MAO-A enzyme is selectively destroyed during solubilization (6–8); lipid extraction with aqueous methylethyl ketone also inactivates MAO-A enzyme whereas MAO-B is relatively intact (7, 9). These MAO-A specific transition temperatures could be attributed to the fact that MAO-A enzyme is more dependent on its lipid environment within the membrane. At least three temperature-induced functional states characterized by differential temperature coefficients were present in membrane-bound MAO-A. The same conclusion was obtained from our previous study using solubilized crude purified MAO-A and MAO-B fractions and a spin-labeled competitive reversible inhibitor (8).

The physical state of the lipid-bilayer domain of the outer mitochondrial membrane, the loci of action of the MAO enzymes, was investigated using spin-labeled

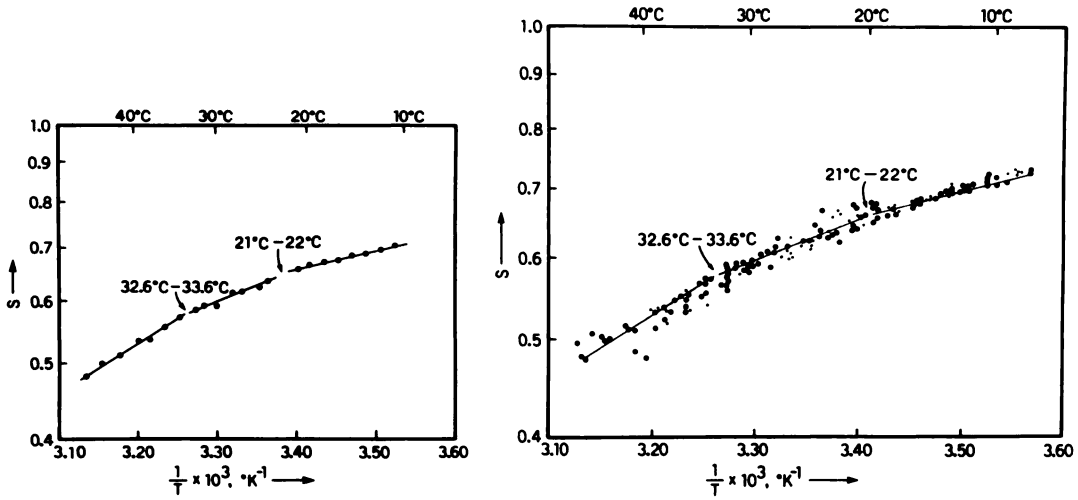


FIG. 4. *Order parameter S calculated from Eq. [1] using I (12, 3) incorporated into the outer membranes of brain mitochondria vs 1/T*
The inset illustrates data from 10 independent experiments. • represents one datum point. ● represents two coinciding datum points.

stearic acids. The phase transition temperature at 29–30° observed using I (1, 14) as a probe reporting the fluidity of the center region of the membrane did not coincide with any of the transition temperatures characterizing the functional states of monoamine oxidase. Apparently, the phase transition that occurred in the center of the bilayer did not influence the conformation of the membrane-bound MAO (Fig. 6).

Phase transition temperatures at 21–22° and 32.6–33.6° were observed using I (12, 3) as a probe reporting the fluidity of the region more proximal to the surface of the membrane (Fig. 4). In this case, a correlation of transformation of the functional state of MAO-A enzyme and the physical properties of the bulk membrane lipids was observed at 21–22°. The bulk membrane lipids interacted with the active site of the MAO-A effectively in the vicinity of the hydrophobic region proximal to the surface of the membrane. In contrast, the active site of MAO-B was quite independent of the physical properties of the bulk hydrocarbon core domain, although MAO-B required phospholipids for its activity. It is of interest in this regard that O'Brien *et al.* have recently shown, using proton and phosphorus NMR spectroscopy in purified rhodopsin:egg phosphatidylcholine recombinant system, regions of the rhodopsin molecule interact in a manner that affects the phospholipids from the aqueous interface (25). The additional break at 35–36° associated specifically with MAO-A may be attributed to a temperature-induced conformational change occurring in the bound lipid shell and/or the enzyme protein. The nature of MAO-A enzyme may be regulated by the physical state of a bound lipid shell and the bulk membrane lipids. Ample evidence has suggested that bulk lipids effects may be associated with many membrane cooperative processes (26, 27). Studies of cytochrome oxidase with spin-labeled lipid indicated that the enzyme complex interacted strongly with a shell of lipid molecules (28). Previously, we have consistently obtained similar characteristic transition temperatures of 21–22° and 35–36° in intact mitochondrial preparations when a spin-labeled competitive reversible inhibitor (amphetamine) was employed as an active site directing probe (17). Further, Houslay and Tipton (10) showed both loss of enzymic substrate–inhibitor specificity and identity of liver mitochondrial MAO-A and MAO-B enzyme protein using a

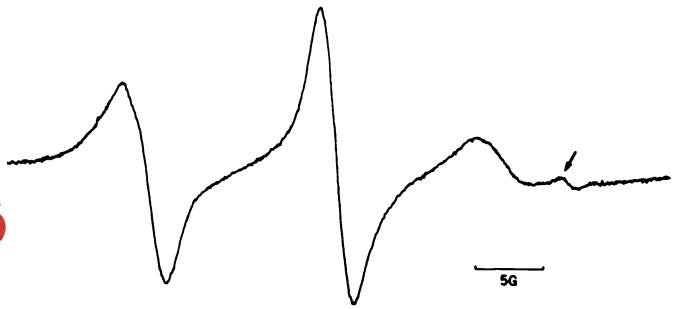


FIG. 5. *Spectrum of I (1, 14) incorporated into the outer membranes of brain mitochondria at 9°*
The arrow indicates signal generated from free tumbling I (1, 14) nitroxide radicals. H_0 = 3490 G.

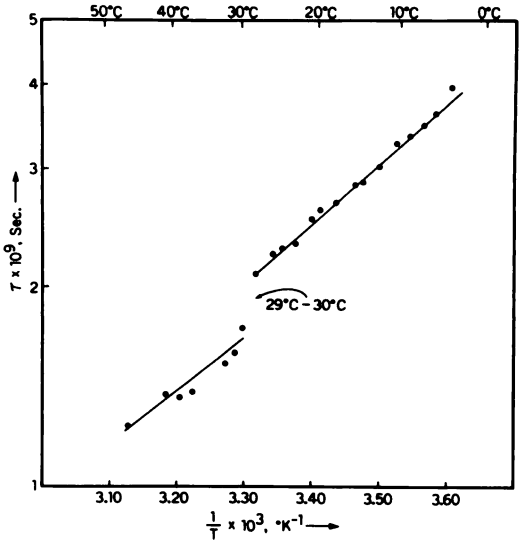


FIG. 6. *Rotational correlation time τ calculated from Eq. [5] using I (1, 14) incorporated into the outer membranes of brain mitochondria vs 1/T*

solubilized preparation treated with chaotropic agents. These observations strongly suggested that MAO-A and MAO-B might be the same protein that existed in different membrane lipid environment. Our study indicated a distinctly different nature of lipid–protein interactions existed for MAO-A and MAO-B *in situ* in an intact membrane preparation. Substrate and inhibitor sensitivities of MAO-A enzyme were modulated in part by the lipid–protein interactions occurring in the region proximal to the membrane surface. In addition, the functional state of MAO-A might be regulated by a tightly bound lipid shell. In contrast, MAO-B appeared to be quite independent of the dynamic properties of the hydrocarbon core of membrane lipids. Our present data could not exclude, unequivocally, the possibility of an intrinsic nonidentity of enzyme proteins either between MAO-A and MAO-B or among the three functional states of MAO-A.

Further studies on the molecular nature of lipid–protein interactions as related to information concerning the multiple functional forms of monoamine oxidase are currently underway.

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Send reprint requests to: Dr. Rosa H. Huang, Department of Biochemistry, College of Medicine, University of South Alabama, Mobile, Ala. 36688.