Significance of Multiple Forms of Brain Monoamine Oxidase in Situ as Probed by Electron Spin Resonance*

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Spin-labeled hydroxyamphetamine, a competitive reversible inhibitor of brain monoamine oxidase, has been shown to be useful as an electron spin resonance (ESR) probe of the microenvironment of the active sites of the possible monoamine oxidase multiple forms. The ESR spectrum of spin-labeled hydroxyamphetamine was strongly quenched upon binding to the enzyme. The conformation of the active site of rat brain monoamine oxidase existing in various physical states, i.e. monoamine oxidase in situ (intact brain mitochondria), crude solubilized monoamine oxidase (MAO) and isolated monoamine oxidase fractions (MAO, and MAO,) were critically and systematically examined. Nonlinear least squares regression analyses have been used to fit the binding data (obtained at room temperature with varying spin-labeled hydroxyamphetamine concentrations) to three groups of independent noninteracting ligand-binding models. A Gibbs-Helmholtz relationship was applied to the interpretation of the measured apparent association constant K as a function of temperature ranging from 4-60° with increments of 2°. From the extracted intensive parameters, k (intrinsic association constant) and ΔF (intrinsic free energy), as well as the apparent heat, ΔH; it was clear that the microenvironment of the binding sites existing in the more purified enzyme fractions MAO, and MAO, were similar to those found in the crude solubilized enzyme. More importantly, they correlated well with the conformation of the sites characterized in situ.

The data suggested that the microenvironment of this multienzyme system was unperturbed in spite of the treatment due to the isolation process. In terms of the composition of binding sites, MAO, appeared to be heterogeneous while MAO, appeared to be more homogeneous. Since the isolated fractions MAO, and MAO, possessed marked different substrate specificities, these observations directly implied that monoamine oxidase multiple forms do exist in situ. The extracted extensive parameters, n (specific binding activity, nanomoles/mg of protein), as well as the measured characteristic transition temperatures, indicated that the relative abundance of the sites which directly affected substrate specificities was indeed altered. The consistency of the characteristic transition temperatures of 21° and 38° for the case of intact membrane preparations was particularly significant. A tenable hypothesis is that the manipulation in the composition of the monoamine oxidase binding forms through intimate lipid-protein interactions, which has been amply demonstrated in many biomembrane systems to be functionally important might be the underlying regulatory mechanism in vivo.

Monoamine oxidase (EC 1.4.3.4) catalyzes the oxidative deamination of the putative neurotransmitters: dopamine, norepinephrine, and serotonin as well as other biogenic amines. Recent studies of monoamine oxidase in vivo and in vitro (1-12) based on indirect evidence derived from multiple substrate and inhibitor studies suggested the possibility of multiple forms. More directly, there have been reports detailing the attempts to isolate the pure enzyme(s) (13-18), and to show the existence of the multiple forms by immunological techniques (19), as well as by the use of differential inhibitors (9). It is generally accepted that monoamine oxidase is located in the outer membrane of the mitochondrion (20-22). The method required for the solubilization of the membrane-bound mitochondrial monoamine oxidase raises the possibility that the observed isolated solubilized monoamine oxidase fractions may be an artifact of the procedure. This uncertainty is enhanced by the report of Houslay and Tipton (23) who have demonstrated that treatment of a rat liver monoamine oxidase...
preparation with a chaotropic agent resulted in the disappearance of the apparent multiplicity of the enzyme both in terms of electrophoretic mobility and substrate specificity.

In previous reports from our laboratory (24), using column chromatographic techniques, we have been able to show that the interconversion among the possible multiple forms of rat brain monoamine oxidase was protein concentration-dependent. More recently, using electron spin resonance techniques (25), we have obtained additional confirmation for these results. In addition, we have also previously reported the use of spin-labeled hydroxyamphetamine, a competitive reversible inhibitor, as an electron spin resonance (ESR) probe of the active site of monoamine oxidase. We have shown that the ESR absorption spectrum of spin-labeled hydroxyamphetamine was strongly quenched when bound to the enzyme and that since the changes in the signal of the probe occurred as a consequence of the enzyme-inhibitor complex, it could be used to quantify the interactions of the probe with the enzyme(s) as well as that of ligands which affect the binding of spin-labeled hydroxyamphetamine. In addition, we have previously demonstrated that spin-labeled hydroxyamphetamine could be employed to measure not only the normality of the active sites of monoamine oxidase but also the composition of the component forms of the multiform enzyme system of monoamine oxidase (25). Since opaque samples can be measured with the ESR technique, it was important and exciting that the conformation of the functional site of an enzyme in situ in the membrane of mitochondria in which that enzyme existed, could be examined critically and systematically with this probe.

In this report then, we wish to report not only the measurements of monoamine oxidase in situ with intact mitochondrial preparations, but also with a crude solubilized preparation, as well as with two different partially purified enzyme fractions. The monoamine oxidase-hydroxyamphetamine-binding characteristics monitored by spin-labeled hydroxyamphetamine were defined using extensive parameters (molar concentration of individual groups of binding sites per mg of protein) as well as extensive parameters ($\Delta F$, intrinsic free energy, $\Delta H$, apparent heat).

**MATERIALS AND METHODS**

**Chemicals**—The chemicals used in this work were obtained as follows: p-hydroxyamphetamine, courtesy of SYVA Corp, Palo Alto, Calif.; clorgyline, courtesy of Dr. J. Shih; 1-deprenyl, courtesy of Dr. J. Knoll, Hungary; $\beta$-[14C]phenylethylamine-hydrochloride, $\beta$-[14C]tryptamine bisuccinate, $\alpha$-[14C]tryptamine hydrobromide, $\beta$-hydroxy$\beta$-tryptamine bixonate from New England Nuclear; (methylene-$\gamma$-C)benzylamine hydrochloride from International Chemical and Nuclear Corp. Other chemicals were obtained from commercial sources.

**Enzyme Preparations**—Intact rat brain mitochondria was prepared by purifying crude mitochondrial fractions from rat brain (male, Sprague-Dawley, weight 180 to 200 g) utilizing a discontinuous Ficoll density gradient (3% and 6%) procedure (26). The preparation (determined by electron microscopy) contained at least 85% intact mitochondria with minute synaptosomal contamination.

Crude solubilized monoamine oxidase (MAO) preparation was derived from rat brain mitochondria according to the procedure of Shih and Eiduson (24). The mitochondria were treated with Triton X-100 followed by 20 to 40% ammonium sulfate fractionation, and then eluted from a calcium phosphate gel.

Isolated enzyme fractions (MAO a, MAO b) were derived from crude solubilized monoamine oxidase preparations, and separated by agarose column chromatography (24).

**Protein concentrations used in the calculations of specific activities were measured by the method of Lowry et al. (27), using bovine serum albumin as standard.**

**Assay of Monoamine Oxidase**—Four different substrates were used to determine monoamine oxidase activity. The isolated enzyme fractions were assayed in duplicate by incubating 0.20-ml aliquots in 1 ml of 0.05 M potassium phosphate buffer, pH 7.4, with 14C-labeled substrates for 30 min at 37°. The method of Robinson et al. (28) was modified when serotonin and tyramine were used as substrates. After incubation, the reaction was stopped and 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 phenylethylamine and tryptamine were substrates for the enzyme (29). The reaction was terminated by adding 0.1 ml of 6 N HCl. Tolueno, 6.0 ml, was then added and the product was extracted and counted in 10 ml of scintillation fluid 2,5-diphenyloxazole (PPO) 1,4-bis(2-[5-phenyloxazolyl])benzene (POPOP), tolue. Total concentration for all substrates was 0.2 mm.

**Tritration and Characterization of Monoamine Oxidase-Spin-labeled p-Hydroxyamphetamine Binding Sites**—Binding sites of the enzyme were characterized using spin-labeled p-hydroxyamphetamine. Binding of spin-labeled hydroxyamphetamine to the active site of the enzyme was assessed by the decrease in signal intensity of the first derivative ESR absorption line, since this signal characterizing the free spin label was strongly quenched when bound to monoamine oxidase (25). All ligand-binding ESR measurements were performed in 1 mmol of 0.14M sodium phosphate buffer, pH 7.4 for immediate use. The solubilized preparation, MAO a, was dissolved in 0.01 M potassium phosphate buffer at pH 7.4 and kept at 4° until used. Lyophilized preparations of MAO a and MAO b (derived from 0.05 M NH4HCO3 buffer at pH 7.4) were buffer at pH 7.4, then redisolved in double glass-distilled water for immediate use.

We assumed a simple but biologically significant model for the multiform monoamine oxidase system, namely, that there were three independent binding sites, S, T, and U, and that no significant allosteric interaction existed (in previous studies, Shih and Eiduson (24, 25) have observed and isolated three separate fractions of monoamine oxidase which had different substrate affinities): $S + I = SI$, $k_e = [SI]/[S][I]$, $T + I = TI$, $k_e = [TI]/[T][I]$, $U + I = UI$, $k_e = [UI]/[U][I]$, $[EI] = [SI] + [TI] + [UI]$, $[E] = [E]_r + [E]_t$. $[E]_r$ represents the concentration of the total enzyme-inhibitor complexes formed. The concentration of the total enzyme-inhibitor complex is represented by [EI] while $[E]_r$ indicates the total enzyme.

The abbreviations used are: MAO a, crude solubilized monoamine oxidase; MAO b, and MAO c, monoamine oxidase-isolated fractions a and b.
normality, $N_e, N_s$ and $N_u$ are the individual enzyme normalities with respect to the ligand spin-labeled hydroxyamphetamine, and $k_e, k_s$, and $k_u$ are the corresponding intrinsic association constants (affinities) which define each individual equilibrium. Thus:

$$[E] = \frac{N_e k_e [I]}{1 + k_e} + \frac{N_s k_s [I]}{1 + k_s} + \frac{N_u k_u [I]}{1 + k_u} \tag{6}$$

(Nonlinear least squares regression analysis, based on the assumption of three independent groups of binding sites, was used to fit the experimental data.) The apparent association constant, $K$ was determined over a temperature range of 4.5 to 52°C by increments of 2°C. The temperature was monitored with a copper-constantan thermocouple and the potential difference between the thermocouple and an ice water reference junction was measured with a high precision potentiometer. E-248-1 variable temperature aqueous solution sample cell was used as sample container. For each enzyme sample, the amount of the enzyme inhibitor complex was measured over the temperature range at several different spin-labeled hydroxyamphetamine concentrations. The temperature at the cell was stable to about ±0.1°C. The incubation time at each temperature prior to the binding measurement was about 20 min. Based on our model of three different binding sites for monoamine oxidase (see Equation 6), the apparent binding constant $K$:

$$K = \frac{[E]}{[E] - [E]} = \frac{1}{\frac{1}{[I]} + \frac{1}{k_e [I]} + \frac{1}{k_s [I]} + \frac{1}{k_u [I]}} \tag{7}$$

Where

$$\alpha_e = \frac{N_e}{E_0} \tag{8}$$
$$\alpha_s = \frac{N_s}{E_0} \tag{9}$$
$$\alpha_u = \frac{N_u}{E_0} \tag{10}$$

$R$ is the gas constant and $T$ is the absolute temperature. From Equation 7 one gets the root of the expanded quadratic equation:

$$[E] = \left[\frac{L_s}{2} + \frac{1}{2K}\right]^2 + \left(\sqrt{\frac{1}{4} - \frac{L_s}{2K}}\right)$$

$$[E]_s = \left[\frac{L_s}{2} + \frac{1}{2K}\right]$$

$K$ increases with temperature (for example, with one intact brain mitochondria preparation at $t = 10^6$ and 48°C, the corresponding $K$ values, were 1.88 x 10^6 M^-1 and 9.65 x 10^5 M^-1, respectively). At the higher temperature (55°C), 1/K approaches zero, and Equation 14 reduces to:

$$[E] = [E]_s \tag{16}$$

Under the condition that $[E]_s > [E]_s$, a good approximation is that the normality of the enzyme, $[E]_s$, is equal to the total concentration of enzyme-inhibitor complex at saturation levels (high temperature limit). Since $[E]$ can be measured directly, the apparent affinity constants at all temperatures can be calculated. The apparent affinity $K$ as a function of temperature was analyzed and interpreted according to the Gibbs-Helmholtz relationship, i.e.

$$\Delta H^\circ = -R \frac{d \ln K}{d \frac{1}{T}} \tag{17}$$

where $\Delta H^\circ$ is the apparent heat that characterized the total equilibrium process.

A competition study was undertaken with the inhibitors, clorgyline or deprenyl, to compete with spin-labeled hydroxyamphetamine for monoamine oxidase binding sites. The normality of spin-labeled hydroxyamphetamine- monoamine oxidase binding was monitored over the temperature range for the intact brain mitochondria sample preincubated with clorgyline or deprenyl at room temperature for 30 min. The $[E]_s$ obtained from a control sample (i.e. intact brain mitochondria without clorgyline or deprenyl treatment) was used for the calculation and analysis.

RESULTS AND DISCUSSION

Measurement of Monoamine Oxidase-Spin-labeled Hydroxyamphetamine Binding as Function of Concentration of Inhibitor (Spin-labeled Hydroxyamphetamine) at Room Temperature — The normality of $[E]$ measured was plotted against the amount of free unbound spin-labeled hydroxyamphetamine in a concentration range of 2 x 10^-4 to 1 x 10^-3 M for enzyme preparations: intact brain mitochondria, MAOa, MAOa, and MAOb (Figs. 1 and 2). The experimental data showed an excellent fit with our basic assumptions (Equation 6) and indicated no marked interactions existed among the sites (illustrated in Fig. 2). Both the intensive and extensive parameters characterizing the enzyme system were extracted from the experimental data and listed in Tables I and II.

It was clear that intact brain mitochondria contained essentially three different inhibitor binding sites, S, T, and U. With respect to the ligand, form U was characterized by an almost irreversible affinity with $\Delta F_u = -80$ kcal. Relatively weak and reversible affinities were observed for forms S and T (Table I): $\Delta F_s = -4.42$ kcal ($k_s = 1.78 \times 10^9$ M^-1) and $\Delta F_r = -6.62$ kcal ($k_r = 7.39 \times 10^9$ M^-1). The relative natural abundance of forms S, T, and U in brain monoamine oxidase in situ was reflected by the ratio of $n_s/n_r/n_u = 16/0.88/1.05$ (Table II).

The crude solubilized MAO preparation also possessed...
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Three groups of binding sites, i.e. form U (ΔF_u = -80 kcal), form S (ΔF_s = -4.12 kcal, k_s = 1.07 × 10^3 M^-1), and form T (ΔF_t = -6.4 kcal, k_t = 5.09 × 10^4 M^-1), shown in Table I. The corresponding relative abundance of forms, S, T, and U in the solubilized preparation was altered from that of the in situ preparation yielding ratios of 180/3.68/0.45 (Table II). These data therefore strongly suggested that during the solubilization process, from intact mitochondria to the crude solubilized preparation, the tertiary structure of the enzyme, as probed by spin-labeled hydroxyamphetamine at the binding sites, remained relatively intact. It was apparent that all of the monoamine oxidase forms that were present in the intact mitochondria were also present in the solubilized preparation. Furthermore, it was observed that the ratios n_s/n_u and n_u/n_t were 0.056 and 0.066, respectively, for monoamine oxidase in situ (Table II). However, the same ratios derived from the solubilized preparation were 0.020 and 0.003 (Table II). It was clear that as the purification of monoamine oxidase proceeded, from the intact to the solubilized preparation, the absolute number of binding sites per mg of protein increased about 11-fold and that the relative ratios of the binding sites were altered. This suggested that the isolation procedure preferentially decreased the relative amounts of "T" and "U".

It was observed that in the isolated fraction MAO_o (with specific activities of 1.85 and 2.30 (nanomoles of substrate oxidized per 30 min per mg of protein) with phenylethylamine and 5-hydroxytryptamine as substrates, respectively) the "S" form was enriched about 7-fold while sites "T" and "U" were only 1/10 and 1/90 that observed for the intact mitochondrial preparation. In the isolated fraction MAO_b (with specific activities of 0.867 and 22.54 using phenylethylamine and 5-hydroxytryptamine as substrates) the "S" form was enriched 55-fold while the "T" form was undetectable. In MAO_o, the ratio n_s/n_u was about 3.6 × 10^-4 as compared to 6.6 × 10^-2 for the intact mitochondrial preparation. Thus in terms of the composition of binding sites, MAO_b was heterogeneous whereas MAO_o appeared to be homogeneous. It was clear that the quality of the binding sites existing in the more purified fractions MAO_o and MAO_b were similar to those observed in the crude solubilized preparation of the enzyme.

More importantly, they correlated well with the conformation of the sites characterized in situ, even though MAO_o and MAO_b showed markedly different substrate specificities (Tables I, II, and III). Thus, it can be stated that the characteristics of the binding sites observed in the isolated fractions MAO_o, MAO_b, and partially purified solubilized MAO_o were in fact found in situ. This direct evidence in turn strongly implied that multiple forms of monoamine oxidase existed in

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**Table I**

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Intrinsic association constant</th>
<th>Intrinsic free energy</th>
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<tr>
<td></td>
<td>k_t</td>
<td>k_s</td>
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<tr>
<td>Brain intact mitochondria</td>
<td></td>
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<tr>
<td>Crude solubilized MAO_a</td>
<td>1.78</td>
<td>7.39</td>
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<td>Isolated fraction, MAO_o</td>
<td>1.07</td>
<td>5.69</td>
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<tr>
<td>Isolated fraction, MAO_b</td>
<td>1.09</td>
<td>1.41</td>
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</table>

* Irreversibly bound.

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**Table II**

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Specific binding activity</th>
<th>Purification</th>
<th>Ratio of binding sites</th>
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<tr>
<td></td>
<td>n_s</td>
<td>n_t</td>
<td>n_u</td>
</tr>
<tr>
<td>Brain intact mitochondria</td>
<td>7.50</td>
<td>16</td>
<td>0.88</td>
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<tr>
<td>Crude solubilized MAO_o</td>
<td>3.75</td>
<td>180</td>
<td>3.68</td>
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<tr>
<td>Isolated enzyme fraction, MAO_o</td>
<td>8.50</td>
<td>107</td>
<td>0.078</td>
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<tr>
<td>Isolated enzyme fraction, MAO_b</td>
<td>1.64</td>
<td>870</td>
<td>0</td>
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* Average of two separate preparations.
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TABLE III

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Protein concentration</th>
<th>Phenylethylamine</th>
<th>Tryptamine</th>
<th>Tyramine</th>
<th>5-Hydroxytryptamine</th>
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<tr>
<td>MAO&lt;sub&gt;a&lt;/sub&gt;</td>
<td>6.05</td>
<td>1.85 ± 0.2</td>
<td>5.4 ± 0.3</td>
<td>4.64 ± 0.35</td>
<td>9.3 ± 0.34</td>
</tr>
<tr>
<td>MAO&lt;sub&gt;b&lt;/sub&gt;</td>
<td>2.34</td>
<td>0.86 ± 0.052</td>
<td>3.51 ± 0.29</td>
<td>1.154 ± 0.206</td>
<td>22.54 ± 0.06</td>
</tr>
</tbody>
</table>

* Specific activity (nanomoles of substrate oxidized/mg protein/30 min). Incubation temperature was 37°C.
* q = (specific activity of 5-hydroxytryptamine)/specific activity of phenylethylamine).
* Duplicate determinations.

The membrane of intact mitochondria, although the ratio of the groups of binding sites were altered by the purification process.

Effect of Temperature on Binding of Inhibitor (Spin-labeled Hydroxymphetamine) to Monoamine Oxidase—The enzyme-inhibitor complex ([EI]/mg of protein) was measured and plotted as a function of temperature (Figs. 3 and 4) for four different monoamine oxidase preparations: i.e. (a) intact mitochondria (12.9 mg of protein/ml); (b) crude solubilized preparation (7.30 mg of protein/ml); (c) isolated MAO<sub>a</sub> (4.70 mg of protein/ml); and (d) isolated MAO<sub>b</sub> (3.70 mg of protein/ml). The spin-labeled hydroxymphetamine concentrations used were 1.03 x 10<sup>-5</sup> M and 1.02 x 10<sup>-6</sup> M for intact brain mitochondria and MAO<sub>a</sub> samples, respectively (Fig. 3). Under this condition it was observed that the amount of the least resistant binding sites (i.e. sites which bound spin-labeled hydroxymphetamine most readily at low temperatures) were 0.07 nmol/mg of protein in MAO<sub>b</sub> and 0.16 nmol/mg of protein in intact brain mitochondria at 20°C. In contrast, the specific binding activities of the binding sites which were activated at higher temperatures were 1.2 nmol/mg of protein in MAO<sub>a</sub> and 0.73 nmol/mg of protein in intact brain mitochondria at 48°C.

In Fig. 4, the amount of nanomoles of inhibitor, spin-labeled hydroxymphetamine, per mg of protein in samples were 6.45 and 8.19 for MAO<sub>a</sub> and MAO<sub>b</sub> respectively. A marked difference in the characteristic ratio of the specific binding activity at 62°C to that at 30°C was observed between MAO<sub>a</sub> (2.0) and MAO<sub>b</sub> (6.0). Interestingly, the corresponding substrate specificity, marked by q, of specific activities with 5-hydroxytryptamine and phenylethylamine as substrates, was 1.25 for MAO<sub>b</sub> and MAO<sub>a</sub> samples, respectively (Table III).

When ln K was plotted against 1/T (Fig. 5) for the intact mitochondria preparation, three straight lines were observed which characterized three distinct binding groups α, β, and γ. The apparent heat associated with each individual binding process was obtained from the slope of the straight line, i.e. ΔH<sub>α</sub> = 16.26 kcal/mol, ΔH<sub>β</sub> = 28.56 kcal/mol, and ΔH<sub>γ</sub> = 65.05 kcal/mol (Table IV). The temperature at which the β form began to dominate over γ form was 21°C. The α form dominated over the β and γ forms at 39°C (Table V). The correlation between the transition temperatures associated with the phenomenon of lateral phase separations of the lipid domain and those associated with the activities of functional proteins in biomembranes has been amply demonstrated in many systems to be functionally important (30-43). The characteristic transition temperatures, 21° and 38°, at which the straight lines intersect (Fig. 5) were particularly significant for the case of intact membrane preparations.

The Gibbs-Helmholtz plot for the crude solubilized monoamine oxidase yielded three intersecting straight lines which were parallel to those obtained from intact brain mitochondria. However, the corresponding transition temperatures for the three slopes were shifted to 17° and 30°. A similar analysis for the isolated fraction MAO<sub>a</sub> showed two straight lines where one line was parallel to the β form and the other was parallel to the γ form with a transition temperature at 34.7°C. In the isolated MAO<sub>b</sub>, the same two forms were observed but the transition temperature was further shifted to 43°C. It was also clear that the change in the ratios of the
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FIG. 5. Gibbs-Helmholtz plot of lnK versus 1/T. □, intact brain mitochondria; *, (MAO) crude solubilized monoamine oxidase; ●, (MAO) monoamine oxidase-isolated fraction a; ○, (MAO) monoamine oxidase-isolated fraction b.

TABLE IV
Apparent heat (ΔH) characterizing binding sites α, β, and γ

<table>
<thead>
<tr>
<th>Site</th>
<th>(ΔH) kcal/mol</th>
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<tbody>
<tr>
<td>α</td>
<td>65.05</td>
</tr>
<tr>
<td>β</td>
<td>28.56</td>
</tr>
<tr>
<td>γ</td>
<td>16.26</td>
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</table>

TABLE V
Transition temperatures (°C)

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>tα → tγ</th>
<th>tβ → tδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact brain mitochondria</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td>Crude solubilized monoamine oxidase</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td>Isolated fraction, MAOa</td>
<td>35</td>
<td>±α</td>
</tr>
<tr>
<td>Isolated fraction, MAOb</td>
<td>43</td>
<td>±α</td>
</tr>
</tbody>
</table>

* + = a form of enzyme not present.

binding forms in MAOa and MAOb reflected a simultaneous shift in substrate specificities (Table III).

Further, competitive binding studies were carried out by monitoring the monoamine oxidase-spin-labeled hydroxyamphetamine binding for the samples and for intact brain mitochondria preincubated with clorgyline (6 × 10⁻⁶ M) and deprenyl (5 × 10⁻⁶ M) for 30 min at room temperature. The Gibbs-Helmholtz plot (Fig. 6) clearly indicated that compared with the control (i.e., binding data obtained with intact brain mitochondria sample alone under the same concentrations of spin-labeled hydroxyamphetamine binding forms γ and β were mostly inhibited by clorgyline (6 × 10⁻⁶ M) while the binding forms of γ and α were almost completely inhibited by pretreatment with 5 × 10⁻⁶ M deprenyl. Many investigators have characterized two forms of monoamine oxidase (A and B). Our data indicated that the binding sites γ and β are equivalent to monoamine oxidase A (the form more sensitive to clorgyline inhibition) while those sites γ and α are equivalent to the B form of the enzyme (the form more sensitive to deprenyl).

The striking parallelism among the straight lines in the Gibbs-Helmholtz plot for data obtained from intact brain mitochondria MAOa, and MAOb and MAO enzyme preparations, further reinforced the notions that the organization and structure of the microenvironment of this multienzyme system was preserved in spite of the treatment during the isolation process, although the relative composition of each individual binding form, which directly affected substrate specificities, were indeed altered.

If the multiple forms of brain monoamine oxidase play a role in regulating the putative neurotransmitters in vivo, it is compelling to believe that direct manipulation of the composition of the monoamine oxidase-binding forms might be the underlying mechanism. It can be speculated that the local lipid moiety associated with the enzyme protein is tightly bound to the protein in such a way as to influence the conformation of the active site. It was clear that this local lipid-protein interaction was relatively insensitive to the physical and chemical perturbations arising from the isolation and purification procedures. Although we have observed shifts in transition temperatures (related in part to the change in quantity of binding sites during purification, perhaps due to the perturbations resulting from the nonionic detergent used in the purification process) we have consistently obtained characteristic transition temperatures of 21° and 38° in different intact mitochondrial preparations when measurements of Vmax as a function of temperature with various substrates were made. These observations strongly suggested that the dynamic properties of the bulk membrane are preparing a manuscript for publication concerning the data mentioned here.
lipid which existed in the intact membrane might regulate the interconversion of binding forms of monoamine oxidase which in turn influences the nature of substrate and inhibitor specificity of monoamine oxidase. It is of interest in this regard, that Shih and Eiduson (24) have shown that extraction of lipid from a mitochondrial preparation resulted in complete loss of enzyme activity in the preparation. Houslay and Tipton (25) also showed loss of enzyme activity and substrate specificity using a liver mitochondrial preparation treated with a chaotropic agent.

Studies on the lipid-protein interaction as related to information concerning the active site of monoamine oxidase is currently underway.

Acknowledgments—We are grateful to Mr. Jeffery Hicks and Mrs. Willow Peng for their skillful assistance. We are also indebted to Dr. David Maxwell, Department of Anatomy, UCLA, for his help in the morphological characterization of intact brain mitochondria with electron microscopy.

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