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SELECTIVE RADIOCHEMICAL LABELING OF TYPES A AND B ACTIVE SITES OF RAT LIVER MONOAMINE OXIDASE

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SUMMARY: By using clorgyline and deprenyl to block the types A and B active sites of monoamine oxidase, respectively, the remaining site could be selectively labeled radiochemically with [^{3}H]pargyline. Titration of the A and B active sites in rat liver mitochondria indicated that they were present in a ratio of 1:3.3. Subunits containing the labeled A and B catalytic sites could not be separated by SDS-gel electrophoresis, and each had a molecular weight of 60,000 daltons.

Pargyline (N-benzyl-N-2-propynylamine) is an irreversible inhibitor of monoamine oxidase (MAO; E.C. 1.4.3.4), forming a covalent adduct with the flavin moiety of the enzyme (1). This reaction appears to be highly specific towards MAO,since [^{14}C]pargyline failed to bind to either other purified flavoproteins (2) or to other proteins when the drug was administered *in vivo* (3). The reaction is stoichiometric with complete inhibition occurring when one mole of inhibitor is bound per mole of flavin. The flavin moiety is covalently bound to the enzyme and is contained in a pentapeptide having the sequence Ser-Gly-Gly-Cys-Tyr which has been isolated from bovine liver MAO (4).

There recently has been considerable evidence for the existence of multiple forms of MAO, based on differences in substrate specificity and inhibitor sensitivity (5). Two forms of MAO (designated as types A and B) can be distinguished by the effects of clorgyline, which selectively inhibits the type A form. A basic question yet to be answered is whether the different types of MAO activity are due to different apoenzymes or to different post-translational modifications (as, for example, by the binding of lipids or other membrane components). This question is not easily answered by using the standard techniques of protein purification, since this enzyme is tightly bound to the

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mitochondrial membrane (6). The techniques needed to solubilize the enzyme, such as the use of sonication and/or detergents, may alter the natural properties of the different enzyme forms by removing them from their lipophilic microenvironment. In addition, the possibility that the observed properties of the solubilized enzyme are due to artifacts caused by incomplete removal of lipids or to residual binding of the detergent cannot be excluded. Moreover, upon purification of MAO, McCauley (7) observed that the enzymatic activity of type A MAO was selectively lost, but he could not rule out the possibility that the purified enzyme preparation contained a catalytically inactive form of the enzyme.

In order to circumvent these problems, we have used [^{3}H] pargyline to label the active sites of MAO in the intact membrane before the protein moieties are solubilized. The A and B sites were selectively labeled by using clorgyline and deprenyl to block the A and B sites, respectively, prior to labeling the remaining site with [^{3}H] pargyline. We have used this approach to titrate the A and B sites and to characterize the labeled subunits by SDS-gel electrophoresis.

MATERIALS AND METHODS. Rat liver mitochondria were isolated according to the procedure of Autilio et al. (8) and resuspended in 0.2M potassium phosphate pH 7.4. MAO activity was assayed by a micro-radiochemical procedure as described by Edwards et al. (9), using [^{14}C]serotonin or [^{14}C]phenylethylamine as substrates. Protein concentrations were determined by the method of Hartree (10), using bovine serum albumin as standard. Electrophoresis was carried out in 7.5% polyacrylamide slab gels in the presence of sodium dodecyl sulfate (SDS), according to the procedure of Weber and Osborn (11). For determination of radioactivity, 2.5 mm gel slices were incubated overnight at 25° in 5 ml of a mixture containing PPO/POPOP/toluene cocktail and 4% Protosol (New England Nuclear). [^{3}H] Pargyline (6.75 Ci/mmole) was prepared by New England Nuclear.

RESULTS: Fig. 1 shows the results obtained from experiments carried out to selectively label either the A or B active sites of MAO with [^{3}H] pargyline. In both cases, the incorporation of the [^{3}H] label increased nonlinearly with a concomitant decrease in enzymatic activity as the amount of added pargyline was increased. In addition, the amount of [^{3}H] pargyline bound appeared to reach a plateau when the amount of pargyline added produced complete enzymatic inhibition. By replotting these data, we observed that the per cent activity decreased linearly with the amount of [^{3}H] pargyline bound (see insets, Fig.1). From these data, the molar quantities of the A and B active sites in the homog-

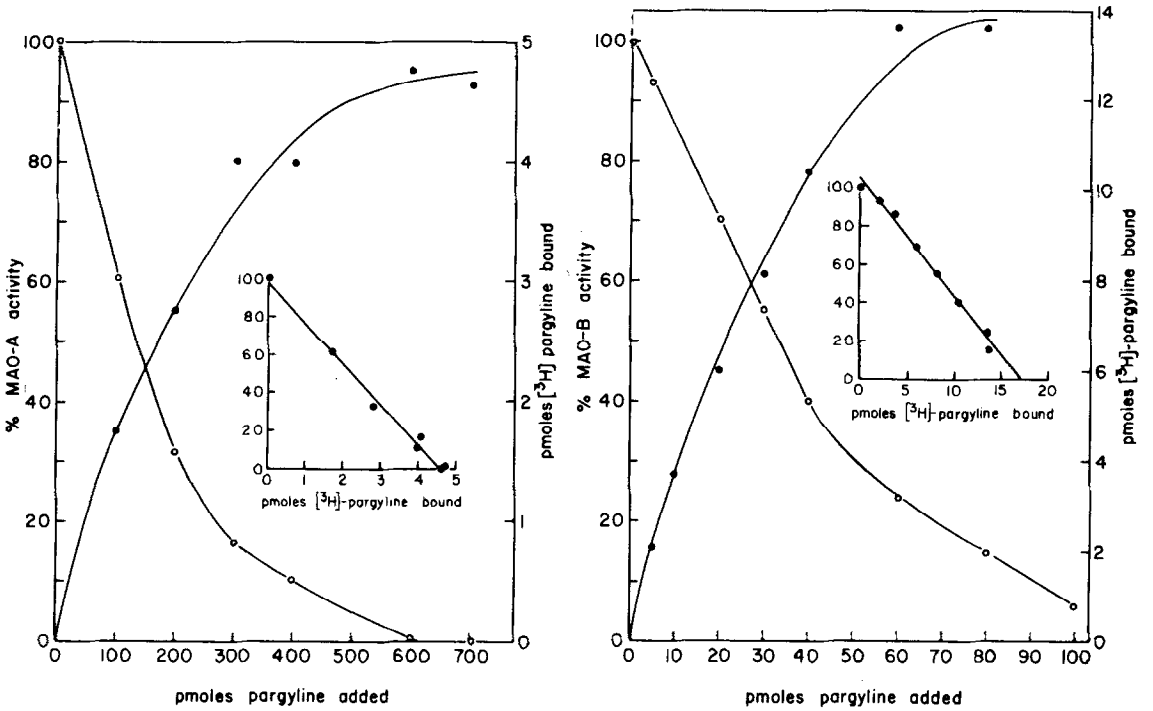

Figure 1. Binding of [^{3}H]pargyline to rat liver mitochondria and the inactivation of the types A and B MAO activities. The specific activities were 91 and 105 nmoles/hr/mg protein when these activities were measured with the substrates, serotonin and phenylethylamine, respectively. Left: Pargyline binding and inactivation of MAO-A. Rat liver mitochondria (containing 227 μg of protein in a volume of 20 μl) were preincubated with 10 μl of 10^{-5} M deprenyl for 40 minutes at 25°. After this treatment, 5% of type B MAO activity and 75% of type A MAO activity were remaining. For the binding studies, [^{3}H] pargyline in amounts ranging from 100 to 700 pmoles was added to give a total volume of 100 μl and incubated for one hr at 25°. The labeled mitochondria were then washed four times with 6% perchloric acid. The final pellet was dissolved in 200 μl of Protosol and the amount of radioactivity was determined by scintillation counting. The amount of pargyline bound was corrected for nonspecific binding of [^{3}H] pargyline by carrying out the labeling with additional samples which had been pretreated with 2 mM unlabeled pargyline. Enzyme activity was monitored in a separate set of tubes treated as above except that unlabeled pargyline was used. Inset: Data were replotted as per cent activity vs. pmoles pargyline bound. The straight line was drawn by least mean squares analysis and the X-intercept was calculated to be 17.0 pmoles (correlation coefficient; r=.994). Right: Pargyline binding and inactivation of MAO-B. These experiments were carried out as above, except 10^{-5}M clorgyline was used instead of deprenyl in order to selectively block type A MAO sites. After this treatment, 83% of type B MAO activity and 0.5% of type A MAO activity was remaining. The B active sites were subsequently labeled with [^{3}H] pargyline by incubation with amounts ranging from 5 to 100 pmoles. Inset: Data were plotted as indicated above; the X-intercept was 4.6 pmoles (r=.995).

enate were calculated (after correction for the amount inhibition of MAO-A by deprenyl and of MAO-B by clorgyline) to be 6.2 and 20.5 pmoles per assay tube or 27 and 90 pmoles/mg protein, respectively. There appears, therefore, to be approximately 3.3 type B active sites for every type A site. The turnover rate of these active sites, calculated from the enzymatic activities measured with serotonin and phenylethylamine under the standard assay conditions, was 3360 hr^{-1} for the A site and 1160 hr^{-1} for the B site.

In order to determine the molecular weight of the subunits containing the A and B catalytic sites by SDS-gel electrophoresis, rat liver mitochondria were pretreated with either clorgyline (to block the type A sites), deprenyl (to block the type B sites) or neither, and the unblocked sites were subsequently labeled with [^{3}H] pargyline. In order to correct for nonspecific binding, control samples were preincubated with high concentrations (10^{-3} M) of deprenyl which block both the A and B sites before the [^{3}H] pargyline was added. As shown in Fig. 2 (left), when the active sites were labeled with 2 x 10^{-6} M [^{3}H] pargyline, two radiolabeled bands were separated by SDS-gel electrophoresis. One band had a mobility slightly faster than the tracking dye; this band appeared even when all MAO activity had been inhibited prior to the addition of [^{3}H] pargyline. The cause of this band is unknown. The other band migrates with an apparent M.W. of 60,000 daltons. Only one band, at this same M.W., was observed whether only the A site, the B site, or both sites had been labeled. However, since at these low concentrations of pargyline the A sites were not fully labeled, these experiments were repeated using a higher concentration of [^{3}H] pargyline (10^{-4} M). This resulted in a greater incorporation of [^{3}H] label into the type A site, as evident by the size of the band at 60,000 daltons. On the other hand, this concentration of pargyline greatly increased the nonspecific binding to other proteins.

DISCUSSION: The present results show that pargyline can be used as a probe to study the multiplicity of active sites of MAO in rat liver. By using clorgyline

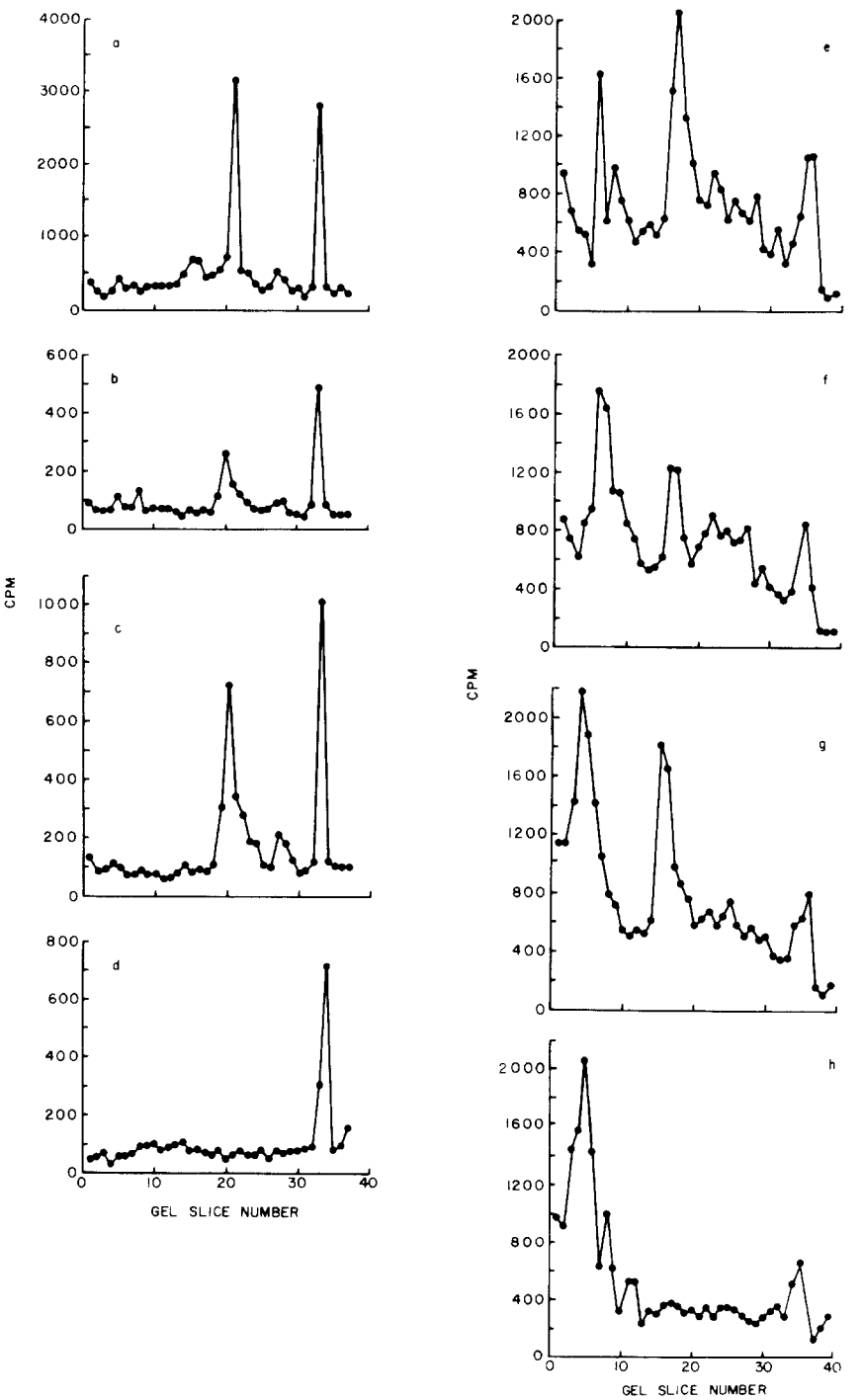

Figure 2. SDS slab gel electrophoresis of rat liver mitochondrial proteins labeled with either 2 x 10^{-6} M [^{3}H] pargyline (left panel, a-d) or 10^{-4} M [^{3}H]pargyline (right panel, e-h). Mitochondria (containing 227 µg of protein) were labeled by incubation in the

and deprenyl to selectively block the A and B sites, respectively, pargyline could be used to titrate or to radiochemically label the other catalytic site.

In our titration studies, we observed that both the types A and B enzymatic activities decreased nonlinearly as an increasing amount of pargyline was added (Fig. 1). In each case, the inactivation curves contained two apparently linear portions of differing slopes, suggesting that pargyline binds with different affinities to separate sites on each of the A or B enzymes; i.e., the inhibitor appears to bind with negative cooperativity to each enzyme. These results are consistent with our previous studies of type B MAO in human platelets in which we found evidence based on substrate binding parameters for two interacting catalytic sites on the enzyme (12). However, in contrast to the present studies, Chuang et al. (13) titrated purified bovine kidney MAO with pargyline and observed a linear decrease in activity with an increase in the amount of inhibitor added. The cause for the discrepancy between these two studies may be due to differences between the solubilized, purified enzyme and the enzyme bound to its natural membrane environment. Alternatively, this discrepancy could be due to differences in the properties of the enzyme in these two species.

The results of the titration studies (as determined by the amount of pargyline bound) show that the concentration of the A and B active sites in liver mitochondria are unequal and are apparently not stoichiometrically related. This argues against the possibility that these two enzymatic activities are due to the presence of separate active sites on the same molecule. Although the specific enzymatic activities of rat liver MAO towards the substrates serotonin and phenyl

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presence of [^{3}H]pargyline, for one hour at 25°. The labeled mitochondria were washed four times with 6% perchloric acid and resuspended in 100 µl of 50 mM Tris buffer, pH 7.4, which contained 1% SDS, 10 mM mercaptoethanol and 0.003% bromophenol blue. One drop of glycerol was added and the samples were heated at 60° for 40 min. Then 50 µl of each sample was subjected to SDS polyacrylamide gel electrophoresis and slices were counted for radioactivity as described in the text. The gels represent samples in which the following active sites were selectively labeled with [^{3}H] pargyline following preincubation with the appropriate selective inhibitor, clorgyline or deprenyl: a and e, both A and B sites; b and f, A site; c and g, B site; d and h, neither site.

ethylamine are similar, our results show that the B and A active sites are present in a ratio of approximately 3.3:1. However, the turnover rate of the A sites appears to be approximately 3-fold higher than the B sites. It should be pointed out that the turnover rates calculated in our studies are approximately 10-fold lower than that calculated previously by other investigators using purified bovine kidney MAO (13). This difference may be due to differences in the substrate used (Chuang et al. [13] used benzylamine) or to differences in the bovine kidney enzyme. (Bovine tissues apparently do not contain multiple forms of MAO [14].)

Although MAO has a low turnover rate, it comprises a surprisingly large proportion of the mitochondrial protein for an enzyme. From the data obtained from the titration studies (pmoles of active site) plus SDS-gel electrophoresis (M.W. of subunit), we can calculate that the A and B enzyme subunits represent 0.16% and 0.54%, respectively, of the total mitochondrial protein. Assuming that rat liver MAO has a molecular weight of 150,000 and consists of two subunits, only one of which contains a catalytic site (15), the enzyme could comprise twice that proportion of the mitochondrial protein. Moreover, since MAO appears to be located primarily on the outer mitochondrial membrane (6), which consists of only about 10% of the total mitochondrial protein (16), it may, therefore, account for about 14% of the protein content of rat liver outer mitochondrial membranes. Similarly, McCauley (17) estimated that rat liver MAO represents about 6% of the outer mitochondrial protein, but it is not clear from his results whether only the B enzyme or both enzyme forms had been labeled.

The molecular basis for the multiple activities of MAO remains unknown. Our results using SDS gel electrophoresis to examine the characteristics of MAO labeled at either the A or B active sites suggest that these two catalytic sites occur on either the same subunit or on separate subunits of equal or almost equal molecular weight. The latter possibility appears to be more likely since the two active sites are present in unequal numbers. Still to be resolved is

whether the A and B enzymes differ with respect to post-translational modifications or whether they represent separate proteins of differing primary structure.

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