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Characterization of the Binding of [^{3}H]Ro 41-1049 to the Active Site of Human Monoamine Oxidase-A

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SUMMARY

The novel reversible and selective inhibitor of monoamine oxidase-A (MAO-A) Ro 41-1049 [*N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide HCl] shows inhibition characteristics similar to those of the structurally related reversible MAO-B inhibitors Ro 16-6491 and Ro 19-6327. In the present study, tritiated Ro 41-1049 was used as a high affinity ligand to study the binding characteristics of this inhibitor to MAO-A and its interactions with the enzyme. An homogeneous population of high affinity binding sites for [^{3}H]Ro 41-1049 was found in membrane preparations from human frontal cortex and placenta (K_d = 16.5 ± 1.4 and 64.4 ± 19.2 nM, respectively). In frontal cortex the B_{max} value for [^{3}H]Ro 41-1049 (2.6 ± 0.4 pmol/mg of protein) was about one third of the B_{max} calculated for the MAO-B-selective ligand [^{3}H]Ro 16-6491. The density of [^{3}H]Ro 41-1049 binding sites in human placenta varied greatly in the different tissue samples investigated, showing an average B_{max} of 101.7 ± 36.5 pmol/mg of protein. Apparent binding equilibrium was reached after 1 hr of incubation at 37°. At this temperature the binding was reversible, with a dissociation $t_{1/2}$ of about 35 min. At lower temperatures the radioactivity dissociation was much slower. Among the various drugs tested, only inhibitors of MAO-A were able to effectively prevent [^{3}H]Ro 41-1049 specific binding. As previously reported for the MAO-B ligands [^{3}H]Ro 16-6491 and [^{3}H]Ro 19-6327, the analysis of the membrane-bound radioactivity showed that [^{3}H]Ro 41-1049 was entirely recovered in the form of its aldehyde derivative, indicating that Ro 41-1049 was deaminated by MAO-A. The existence of a Ro 41-1049 adduct reversibly bound to the enzyme active site might explain the inhibition mechanism of this compound. The exposure of the radioligand-enzyme complex to $NaBH_3CN$ at pH 4.5 caused the irreversible covalent incorporation of about 70% of the specifically bound radioactivity into a 60-kDa polypeptide. This incorporation was dependent on the pH and on the amount of $NaBH_3CN$ added. The presence of MAO-A- but not MAO-B-selective inhibitors prevented the covalent incorporation of [^{3}H]Ro 41-1049. The present results indicate that [^{3}H]Ro 41-1049 is incorporated into a subunit of MAO-A, in the presence of $NaBH_3CN$, and modifies a protein domain that is essential for the enzyme activity. Proteolysis experiments of MAO-A affinity labeled with [^{3}H]Ro 41-1049 or with [^{3}H]pargyline, a drug that forms a stable adduct with the enzyme cofactor FAD, suggest that [^{3}H]Ro 41-1049 is incorporated into a protein fragment of MAO-A containing FAD.

Mitochondrial MAO (EC 1.4.3.4) is present in two distinct forms, namely MAO-A and MAO-B. Both isoenzymes, which have covalently bound FAD as coenzyme (1, 2), catalyze the oxidative deamination of various neurotransmitter amines like catecholamines and 5-hydroxytryptamine (serotonin) as well as of other endogenous or exogenous monoamines. The two MAO isoenzymes have been originally distinguished by their different pharmacological specificities towards substrates and inhibitors (3, 4) and subsequently by their different biochemical and immunological properties (5–7). Recently, several reports have shown that MAO-A and MAO-B differ in primary structure (8–10), with about 70% homology, and are coded by distinct genes, both located on the X chromosome (11–13). MAO-A and MAO-B differ in distribution in various central nervous system regions and in peripheral organs and the ratio between the two forms varies in different animal species (14–16).

Each MAO form is believed to represent a selective target in the treatment of various psychiatric and neurological disorders (17, 18). MAO-A inhibitors are used in the therapy of depression (4), whereas MAO-B inhibitors are under clinical trial for prevention and adjuvant treatment of Parkinson's disease (19).

Labeled MAO inhibitors selective toward either form of the enzyme represent very useful probes for the precise localization and titration of MAO-A and MAO-B in various tissues and

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ABBREVIATIONS: MAO, monoamine oxidase; Ro 16-6491, *N*-(2-aminoethyl)-*p*-chlorobenzamide HCl; Ro 19-6327, *N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide HCl; Ro 41-1049, *N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide HCl; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography, T, total acrylamide concentration; C, crosslinker concentration.

brain areas as well as for the study of the biochemical characteristics of the enzyme. Thus, [³H]harmaline has been successfully employed as ligand for MAO-A investigations (20) and, more recently, we have described the binding characteristics of the reversible and selective MAO-B inhibitors [³H]Ro 16-6491 (21) and [³H]Ro 19-6327 (22, 23). As binding ligands, [³H]Ro 16-6491 and [³H]Ro 19-6327 show many interesting features. They bind selectively to MAO-B and do not show affinity for the A form or for receptors and carrier systems of monoamine neurotransmitters (24, 25).

Ro 16-6401 and Ro 19-6327 behave as reversible mechanism-based inhibitors of MAO-B, being transformed by the enzyme itself into a not yet identified adduct, which appears to be tightly but reversibly bound to the enzyme active site. We have also found that the reduction of the inhibitor-enzyme complex with $NaBH_3CN$ at acidic pH results in the covalent irreversible incorporation of [³H]Ro 16-6491 and [³H]Ro 19-6327 into MAO-B, thus permitting the selective affinity labeling of the protein (25, 26).

Recently, by modifying the aromatic part of these MAO-B inhibitors, it was possible to obtain a new compound, Ro 41-1049, with MAO-inhibiting characteristics similar to those of Ro 16-6491 and Ro 19-6327 but with a remarkable selectivity towards MAO-A (27, 28).

In the present study we describe the synthesis of tritiated Ro 41-1049 and its use as a biochemical tool for the characterization of MAO-A.

Materials and Methods

Chemicals

Ro 41-1049, Ro 41-8328, Ro 43-6329, Ro 19-6327, Ro 16-6491 (for structures see Figs. 1 and 10), MPTP, and MPP^+ were synthesized at Hoffmann-La Roche (Basel, Switzerland). The following drugs were used: selegiline HCl (I-deprenyl; Research Biochemicals Inc., Natick, MA), clorgyline HCl (May & Baker, London, UK), harmaline HCl (Fluka, Buchs, Switzerland), and pargyline HCl (Sigma, St. Louis, MO). [³H]Ro 16-6491 (specific activity, 622.4 GBq/mmol) was kindly prepared by Dr. N. Flück at Hoffmann-La Roche (Basel). All other chemicals were of analytical grade and were obtained from commercial sources.

Spectra were recorded with the following instruments: ¹H NMR spectra [δ ppm) relative to internal tetramethyl silane; J in Hz], Varian

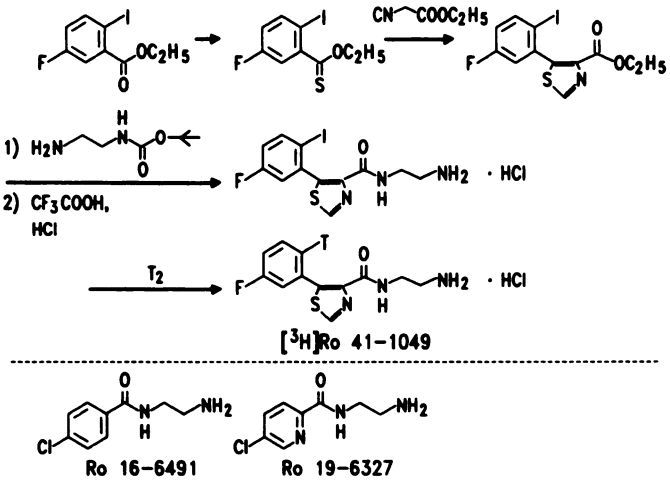

Fig. 1. *Top*, synthesis of [³H]Ro 41-1049. *Bottom*, chemical structure of the MAO-B-selective inhibitors Ro 16-6491 and Ro 19-6327.

EM390 and Bruker AC-250; mass spectra, MS9-ZAB, data system SS 200.

Synthesis of [³H]Ro 41-1049

Ethyl 5-fluoro-2-iodo-thiobenzoate. To a solution of ethyl 5-fluoro-2-iodo-benzoate (8.9 g, 28.7 mmol) in xylene (70 ml) was added 2,4-bis-(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide (7.0 g, 17.3 mmol). After refluxing for 2 days, the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, hexane/toluene 3:1), yielding ethyl 5-fluoro-2-iodothiobenzoate (5 g, 53.3%) as a yellow oil. ¹H NMR (CDC_3: δ 7.83 (dd, J = 6, J = 9, 1 H), 7.36 (dd, J = 3, J = 9, 1 H), 6.90 (dt, J = 3, J = 9, J = 9, 1 H), 4.76 (q, J = 7.5, 2 H), 1.56 (t, J = 7.5, 3 H).

Ethyl 5-(5-fluoro-2-iodophenyl)-4-thiazole carboxylate. To a solution of ethyl 5-fluoro-2-iodothiobenzoate (5 g, 17 mmol) in ethanol (15 ml) were added ethylisocyanoacetate (2.3 g, 20 mmol) and sodium hydroxide powder (500 mg). The mixture was refluxed for 15 hr and partitioned between water and dichloromethane. The organic layer was concentrated and the residue was subjected to column chromatography (silica gel, dichloromethane) to yield ethyl 5-(5-fluoro-2-iodophenyl)-4-thiazole carboxylate (2.9 g, 46%) as a yellow oil. ¹H NMR ($CDCl_3$) δ 8.93 (s, 1 H), 7.94 (dd, J = 6, J = 9, 1 H), 7.15 (dd, J = 3, J = 9, 1 H), 6.93 (dt, J = 3, J = 9, 1 H), 4.27 (q, J = 7.5, 2 H) 1.15 (t, J = 7.5, 3 H).

***N*-(2-Aminoethyl)-5-(5-fluoro-2-iodophenyl)-4-thiazole carboxamide hydrochloride.** Ethyl 5-(5-fluoro-2-iodophenyl)-4-thiazole carboxylate (2.9 g, 7.7 mmol) and *tert*-butyl-(2-aminoethyl)carbonate (4 g, 25 mmol) were heated at 100° for 24 hr. The residue was triturated with ethanol to give a white crystalline residue. To a solution of the crystals in dichloromethane (20 ml), trifluoroacetic acid (5 ml) was added and the solution was refluxed for 1 hr. The mixture was partitioned between 2 N sodium carbonate and dichloromethane and the combined organic layers were subsequently dried over sodium sulfate and concentrated *in vacuo*. The hydrochloride was prepared from the base by treatment with alcoholic hydrogenchloride. Yield, 1.4 g (43.75%); m.p. 270° (decomposition). ¹H NMR (dimethyl sulfoxide): δ 9.28 (s, 1 H), 7.92 (dd, J = 5.8, J = 9, 1 H), 7.36 (dd, J = 2.8, J = 9, 1 H), 7.08 (dt, J = 2.8, J = 9, 1 H), 3.42 (m, 2 H), 2.91 (m, 2 H). Mass spectrum (m/z): 391 (M^+), 362 (M − CH_2NH).

***N*-(2-Aminoethyl)-5-(3-fluorophenyl-6*T*)-4-thiazole carboxamide hydrochloride.** To a solution of *N*-(2-aminoethyl)-5-(5-fluoro-2-iodophenyl)-4-thiazole carboxamide hydrochloride (69 mg, 0.16 mmol) in methanol (4.4 ml) was added palladium/C catalyst (10%, 8 mg). After the addition of methanolic KOH (0.69 ml, 0.46 N), the mixture was stirred in the presence of tritium (4 ml, ~10 Ci). After the absorption of 75%, the reaction was stopped and filtered. The solvent was removed and the residue was subjected to chromatography (silica gel, dichloromethane/2% methanol/3% triethylamine). The hydrochloride was prepared from the base with alcoholic hydrogen chloride. Recrystallization from ethanol gave *N*-(2-aminoethyl)-5-(3-fluorophenyl-6*T*)-4-thiazole carboxamide hydrochloride (18.2 mg, 38%; specific activity, 795 GBq/mmol).

Preparation of Membranes from Human Placenta and Frontal Cortex

Human placentas were frozen within 1 hr after delivery and stored at −70° until processed. After thawing in isotonic saline solution containing 0.5 mM PMSF and 14 mg/liter aprotinine (Trasylol; Bayer, Leverkusen, Germany), the placentas were separated from the amniotic membranes and the connective tissues and cleared as much as possible of blood clots, in order to lower the contamination with MAO-B deriving from blood platelets. The tissue was then cut into small pieces and homogenized in 5 volumes of 0.32 M sucrose buffered to pH 7.4 using 5 mM HEPES (containing 0.5 mM PMSF and 14 mg/liter aprotinine), using an Ultra-Turrax tissue grinder (1 min, setting 10). All the steps were carried out at 4° using ice-cold buffers. The homogenate was centrifuged (800 × g for 20 min), and the supernatant was collected. The sediment was then homogenized and centrifuged a

second time as described above. The two supernatants were mixed and centrifuged at 19,000 × *g* for 20 min. The pellet was resuspended in Tris buffer (50 mM Tris, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4) and subsequently centrifuged (19,000 × *g*, 20 min). This procedure was repeated twice. The membrane preparation was finally resuspended in Tris buffer (8–10 mg of protein/ml) and stored at −70° until analysis.

Human frontal cortices were obtained from three routine autopsy cases (post mortem delay, 24–48 hr) in which no evidence of neurological abnormalities was present. Frontal cortex membranes were prepared as previously described (26) and stored at −70° in Tris buffer, at a protein concentration of 10 mg/ml.

The protein content was determined using the Pierce BCA protein assay kit (Pierce, oud-Bejerland, The Netherlands).

[^{3}H]Ro 41-1049 Reversible Binding

Aliquots of frontal cortex and placenta membrane preparations (0.125 and 0.080 mg of protein/sample, respectively) were incubated for 1 hr at 37° (unless otherwise stated) in Eppendorf 3810 plastic tubes in 300 μl of Tris buffer, containing various concentrations of [^{3}H]Ro 41-1049, with drugs present or absent. After the incubation, 1 ml of cold Tris buffer was added and the tubes were centrifuged in an Eppendorf Microfuge (12,000 × *g* for 3 min). The pellets were then rinsed (1 ml of Tris buffer), solubilized in 10% (w/v) SDS, (2 hr at 60°), and transferred into plastic vials for radioactivity counting.

The nonspecific binding was determined in parallel samples containing 10 μM harmaline.

The reversible binding of the MAO-B ligand [^{3}H]Ro 16-6491 was performed as previously described (24).

HPLC Analysis of Bound and Free Radioactivity

Membrane samples were incubated (1 hr at 37°, unless otherwise stated) in the presence of 50 nM [^{3}H]Ro 41-1049. After membrane sedimentation and rinsing, the radioactivity bound to the membranes was extracted with 200 μl of ethanol containing unlabeled Ro 41-1049 and its aldehyde (Ro 41-8328) and its alcohol (Ro 43-6329) derivatives (0.5 mM each; for structures see Fig. 10). Some samples were extracted with 200 μl of ethanol containing $NaBH_4$ (2 mg/ml, final concentration). All samples were resuspended by sonication (5–10 min in an ultrasonic bath) and centrifuged (5 min at 4°) in an Eppendorf microfuge. Aliquots of the ethanolic extracts (50 μl) were finally injected onto a HPLC reverse phase column (0.5 × 30 cm, Nucleosyl 100-7 C18; Macherey-Nagel, Dueren, Germany). The free radioactivity remaining in the incubation medium was subjected to HPLC analysis by direct injection of a 25-μl aliquot of the medium onto the HPLC column. The samples were eluted at a flow rate of 1 ml/min (mobile phase, 14 g/liter citric acid monohydrate and 5.93 g/liter Na_2HPO_4 dihydrate, pH 3.4, containing 25% ethanol), with the eluate UV absorbance being monitored at 254 nm. Fractions (1 ml) were collected and counted for radioactivity.

Affinity Labeling of MAO-A with [^{3}H]Ro 41-1049

The procedure of affinity labeling MAO-A with [^{3}H]Ro 41-1049 was essentially the same as that used for labeling MAO-B with [^{3}H]Ro 16-6491 (26).

Membrane preparations were incubated for 1 hr at 37° in the presence of various concentrations of [^{3}H]Ro 41-1049. The tubes were then placed at 20°, $NaBH_3CN$ (final concentration, 2 mg/ml unless otherwise indicated) was added, and the pH was adjusted immediately thereafter to 4.5 by the addition of 30 μl of acetic acid (1%, v/v). The incubation was then continued for other 10 min at 20°. After centrifugation and rinsing, the membrane pellet was solubilized (2 hr at 60°) in 300 μl of 0.125 M Tris buffer, pH 6.8, containing SDS (3% w/v) and 2-mercaptoethanol (2% v/v). To separate the radioactivity irreversibly bound to proteins, an aliquot (200 μl) was poured on prepacked Sephadex PD-10 columns (9.1 ml of gel; Pharmacia, Uppsala, Sweden) and then eluted with 0.1 M NaH_2PO_4, pH 6.8, containing 0.1% SDS.

Affinity-labeled membranes were also subjected to SDS-PAGE, 12.5% total acrylamide concentration, 1% crosslinker concentration), according to the method of Laemmli (29). After rapid Coomassie blue staining and destaining, the gels were impregnated with Amplify (Amersham, Little Chalfont, UK) for 45 min at 20°, dried, and finally exposed to Hyperfilm MP (Amersham) at −70° for several days.

[^{3}H]Pargyline Affinity Labeling

The binding experiments with the irreversible MAO inhibitor [^{3}H]pargyline (specific activity 844.7 GBq/mmol; New England Nuclear, Boston, MA) were performed by incubating membrane preparations (1 hr at 37°) in the presence of 100–200 nM labeled compound. Due to the low specificity of [^{3}H]pargyline, the selective labeling of MAO-A and MAO-B was achieved by performing the incubation in the presence of 5 μM Ro 19-6327 or 5 μM harmaline, respectively.

Limited Proteolysis of [^{3}H]Ro 41-1049- and [^{3}H]Pargyline-Affinity Labeled Membranes

Limited proteolysis of placenta MAO-A affinity labeled with [^{3}H]Ro 41-1049 and [^{3}H]pargyline was performed according to the method of Cleveland *et al.* (30). Labeled membranes were subjected to a first SDS-PAGE step as above. After rapid staining and destaining, the gels were sliced in correspondence to the molecular mass of MAO-A (58–62 kDa). The gel slices were equilibrated for 1 hr in 0.125 M Tris buffer, pH 6.8, containing 1 mM EDTA and 0.1% SDS. Labeled MAO-A was then subjected to a second SDS-PAGE step on a 10% T/3% C gel, prepared according to the method of Schaegger and von Jagow (31), by placing the slices directly in the wells of the stacking gel (4% total acrylamide concentration, 3% crosslinker concentration, pH 7.5). Various amounts of *Staphylococcus aureus* V8 protease were added to the gel wells. A constant voltage of 40 V was applied for about 90 min until the samples had entered into the stacking gel. After the power was turned off for 1 hr, electrophoresis was performed at a constant voltage of 130 V. Coomassie blue-stained gels were then treated for fluorography as described above.

Trypsin Proteolysis of Affinity Labeled Purified MAO-A

MAO-A purified from human placenta (kindly supplied by Dr. C. W. Abell, The University of Texas at Austin, Austin, TX) was affinity labeled with 100 nM [^{3}H]Ro 41-1049 as described above and, in parallel samples, with 200 nM [^{3}H]pargyline (80 min at 37°). After dialysis against 6 M guanidine HCl in 50 mM NH_4HCO_3, MAO-A was carboxymethylated by two additions of 20 mM iodoacetamide and then further dialyzed against 50 mM NH_4HCO_3 containing 2 M urea. This MAO-A preparation was incubated with trypsin (enzyme to substrate ratio, 1:20) for 18 hr at 37°. The incubation was stopped by addition of sample buffer and boiling. The digested samples were finally electrophoresed on a 16% total acrylamide concentration/3% crosslinker concentration gel (30). After staining and destaining, the gel was treated for fluorography as above.

Binding Calculations

Binding kinetic data were calculated using a computer program (32).

Results

Reversible binding of [^{3}H]Ro 41-1049. The specific binding of [^{3}H]Ro 41-1049 (50 nM) to human frontal cortex (Fig. 2) and placenta membrane preparations reached apparent equilibrium after about 1 hr of incubation at 37°. At 20°, the binding to frontal cortex membranes was slower, reaching equilibrium after 3 hr (Fig. 2). [^{3}H]Ro 41-1049 bound specifically also after incubation at 4°, but the binding was much lower, increasing very slowly with time of incubation. At 37°, bound radioactivity dissociated from brain membranes after the addition of 10 μM harmaline with a $t_{1/2}$ of about 35 min

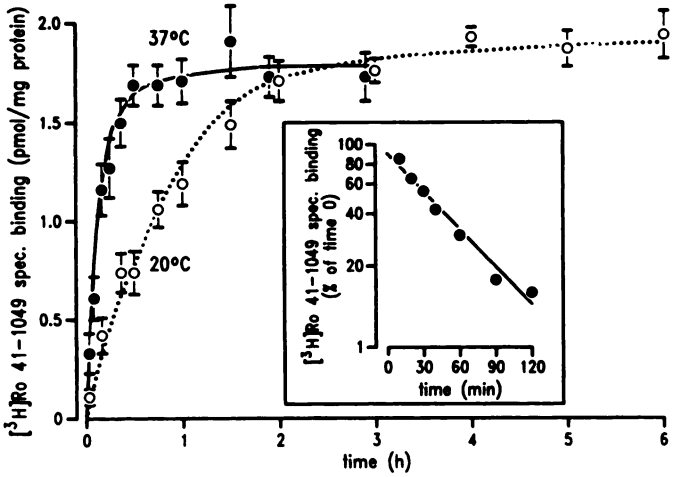

Fig. 2. Time course of [³H]Ro 41-1049 (50 nM) specific binding to human frontal cortex membranes incubated at 37° (●) or at 20° (○). *Points* are means ± standard errors of three experiments performed in duplicate. *Inset*, radioactivity dissociation from human frontal cortex membranes at 37°. The preincubation was performed in the presence of 50 nM [³H]Ro 41-1049 for 1 hr at 37°. At time 0, harmaline (10 μM) was added to the incubation mixture. Aliquots (300 μl) were sampled at the indicated times and treated as described in Materials and Methods.

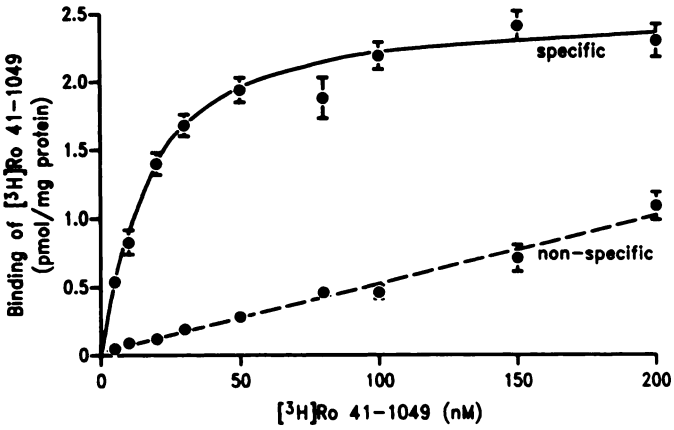

Fig. 3. Equilibrium binding of [³H]Ro 41-1049 to human frontal cortex membranes at various ligand concentrations at 37°. *Points* are means ± standard errors of three experiments performed in duplicate.

(Fig. 2, *inset*), whereas, at lower temperatures (20° and 0°), little radioactivity dissociation was observed after up to 7 hr of incubation (not shown).

Fig. 3 shows the saturation curve of [³H]Ro 41-1049 specific binding as observed in brain membranes at 37°. The binding was of high affinity (K_d = 16.5 ± 1.4 nM), with a B_{max} of 2.6 ± 0.4 pmol/mg of protein. Slightly higher K_d and B_{max} values were measured when the incubation was performed at 20° (30.2 ± 2.4 nM and 3.1 ± 0.6 pmol/mg of protein, respectively). In the same brain membrane preparations, the binding of the selective MAO-B ligand [³H]Ro 16-6491 showed a B_{max} of 8.0 ± 1.3 pmol/mg of protein, indicating that the MAO-B/MAO-A ratio was about 3 in these preparations.

[³H]Ro 41-1049 binding at 37° displayed a somewhat lower affinity (K_d = 64.4 ± 19.2 nM) in human placenta than in brain membranes, and the number of sites labeled varied greatly in the preparations obtained from four different placentae (average B_{max} = 101.7 ± 36.5 pmol/mg of protein).

In the different tissue preparations, a good correlation was found between the [³H]Ro 41-1049 B_{max} values and the MAO-A activity measured radioenzymatically using [¹⁴C]5-hydroxytryptamine as substrate (33) (data not shown).

Scatchard transformation of the saturation curves indicated that a homogeneous population of binding sites was labeled by [³H]Ro 41-1049 both in frontal cortex and in placenta membranes.

Inhibition of [³H]Ro 41-1049 reversible binding. Table 1 shows the effect of various inhibitors or substrates for MAO on the binding of [³H]Ro 41-1049. Among the various inhibitors used, only MAO-A blockers were able to effectively prevent [³H]Ro 41-1049 binding, whereas MAO-B inhibitors were much less potent. The dopaminergic neurotoxin MPTP, which is known to be a substrate for both forms of MAO (34), was able to prevent the binding of [³H]Ro 41-1049. MPP^+, the active metabolite into which MPTP is converted by MAO and which has been shown to bind to MAO-A with a relatively high affinity (35), showed a higher potency than MPTP in inhibiting [³H]Ro 41-1049 binding.

The Hill coefficient of the competition curves for all the drugs tested was close to unity (Table 1).

HPLC analysis of bound and free radioactivity. HPLC analysis of the radioactivity bound to frontal cortex membranes after [³H]Ro 41-1049 binding shows that the bulk of the radioactivity was isographic with the deaminated aldehyde derivative of Ro 41-1049 and only a minor amount coeluted with the original compound (Fig. 4A). The presence of 10 μM harmaline abolished the aldehyde peak without affecting the amount of radioactivity coeluted with Ro 41-1049 (not shown), indicating that, most probably, unmodified [³H]Ro 41-1049 represented nonspecific binding. When the radioactivity was extracted in the presence of the reducing agent $NaBH_4$, the aldehyde peak disappeared and the radioactivity coeluted with the alcohol derivative Ro 43-6329 (Fig. 4A). The radioactivity elution pattern showed similar characteristics whether the incubation was carried out at 20° or at 0°.

On the other hand, the bulk of the radioactivity present in the incubation medium coeluted with [³H]Ro 41-1049 after incubation (1 hr at 37°) (Fig. 4B). Similar results were obtained for longer incubation time (up to 3 hr at 37°; not shown).

Irreversible affinity labeling of MAO-A with [³H]Ro 41-1049. By addition of the mild reducing agent $NaBH_3CN$ to the [³H]Ro 41-1049 membrane complex and lowering of the pH of the sample to 4.5 with acetic acid, part of the specifically

TABLE 1

Effect of various drugs on the binding of [³H]Ro 41-1049 to human brain (frontal cortex) and placenta membrane preparations

The [³H]Ro 41-1049 concentration was 50 nM. IC_{50} values (concentration of drugs causing 50% inhibition of specific binding) and Hill coefficients (n_H) were calculated from competition experiments using at least seven different drug concentrations. Values are means ± standard errors of three experiments.

Drug	Frontal cortex		Placenta	
	IC_{50}	n_H	IC_{50}	n_H
	M		m	
Ro 41-1049	$5.3 \pm 0.4 \times 10^{-8}$	0.8	$6.5 \pm 1.5 \times 10^{-8}$	1.0
Harmaline	$1.0 \pm 0.2 \times 10^{-6}$	1.0	$1.2 \pm 0.3 \times 10^{-6}$	1.1
Clorgyline	$7.2 \pm 1.3 \times 10^{-8}$	1.2	$1.8 \pm 0.3 \times 10^{-8}$	1.3
Selegiline	$1.8 \pm 0.4 \times 10^{-6}$	1.2	$7.4 \pm 1.8 \times 10^{-6}$	1.2
Pargyline	ND*		$3.1 \pm 0.2 \times 10^{-6}$	1.2
Ro 19-6327	$3.3 \pm 0.4 \times 10^{-4}$	1.3	ND	
MPTP	ND		$2.0 \pm 0.3 \times 10^{-5}$	0.9
MPP^+	ND		$5.6 \pm 1.3 \times 10^{-6}$	1.1

* ND, not determined.

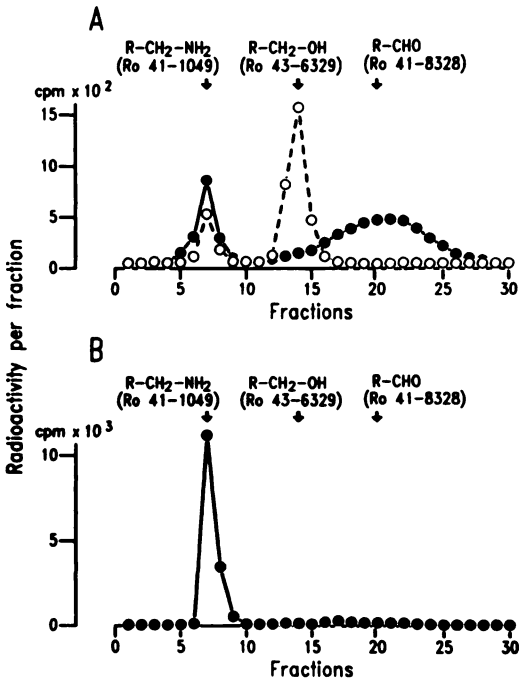

Fig. 4. A, Reverse phase HPLC analysis of radioactivity bound to human frontal cortex membranes incubated for 1 hr at 37° with 50 nM [^{3}H]Ro 41-1049. Bound radioactivity was extracted in ethanol with (○) or without (●) $NaBH_4$ (2 mg/ml). Samples were eluted as described in Materials and Methods. The elution position of Ro 41-1049 and its aldehyde (Ro 41-8328) and alcohol (Ro 43-6329) derivatives are indicated by *arrows*. B, HPLC analysis of free radioactivity in the incubation medium after binding of 50 nM [^{3}H]Ro 41-1049 to frontal cortex membranes for 1 hr at 37°.

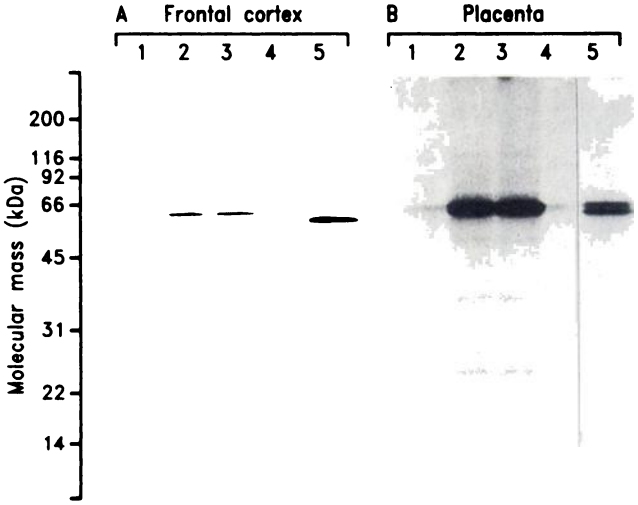

Fig. 5. Fluorogram of gels after SDS-PAGE (12.5% total acrylamide concentration, 1% crosslinker concentration) of [^{3}H]Ro 41-1049-affinity labeled frontal cortex and placenta membrane preparations. Membranes were incubated in the presence of 100 nM [^{3}H]Ro 41-1049 (1 hr at 37°), with or without subsequent treatment (for 10 min at 20°) with 2 mg of $NaBH_3CN$/ml and 1% acetic acid to pH 4.5. *Lane 1*, control; *lane 2*, $NaBH_3CN$ plus acetic acid; *Lane 3*, as before plus 5 μM Ro 19-6327; *lane 4*, plus 5 μM harmaline; *lane 5*, affinity labeling with 200 nM [^{3}H] pargyline (80 min at 37°). The molecular mass scale (kDa) given in the *ordinate* was obtained by using the following marker proteins: myosin (200), β-galactosidase (116), phosphorilase B (92), albumin (66), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (22), lysozyme (14).

bound radioactivity became irreversibly bound to the membrane. SDS-PAGE analysis of the irreversibly labeled membranes showed that, after the reduction step, the radioactivity was incorporated into a single protein band of 60 kDa, using either brain or placenta membranes (Fig. 5). The presence of the MAO-A inhibitor harmaline (10 μM) completely prevented the radioactivity incorporation. No labeling was observed in the absence of $NaBH_3CN$ or in presence of $NaBH_3CN$ when the pH was not adjusted to 4.5. In other experiments with human brain, [^{3}H]pargyline, an irreversible MAO inhibitor that preferentially inhibits MAO-B (4) but displays, however, some affinity for the A isoenzyme, labeled a polypeptide of 58 kDa (corresponding to MAO-B) (25, 36), whereas in placenta membrane preparations [^{3}H]pargyline was incorporated into two bands with molecular masses of 58 and 60 kDa. In placenta membranes, harmaline (2 μM) abolished the 60-kDa band, whereas Ro 19-6327 (2 μM) prevented the labeling of the 58-kDa band (not shown).

As shown in Fig. 6A, the optimal pH of the affinity labeling reaction appeared to be around 4.5, and at this pH the maximal radioactivity incorporation was observed with the addition of 2 mg/ml reducing agent (Fig. 6B). The labeling reaction was very fast, being already maximal after 5 min of exposure to $NaBH_3CN$ at pH 4.5.

In these optimal labeling conditions, about 70% of the [^{3}H] Ro 41-1049 specifically bound was irreversibly incorporated into placenta MAO-A (Fig. 7). The reversible binding yielded a B_{max} of 158 ± 13 pmol/mg of protein, whereas the B_{max} of the irreversible binding was 109 ± 10 pmol/mg of protein.

After exposure of placenta membranes to 1 μM unlabeled Ro

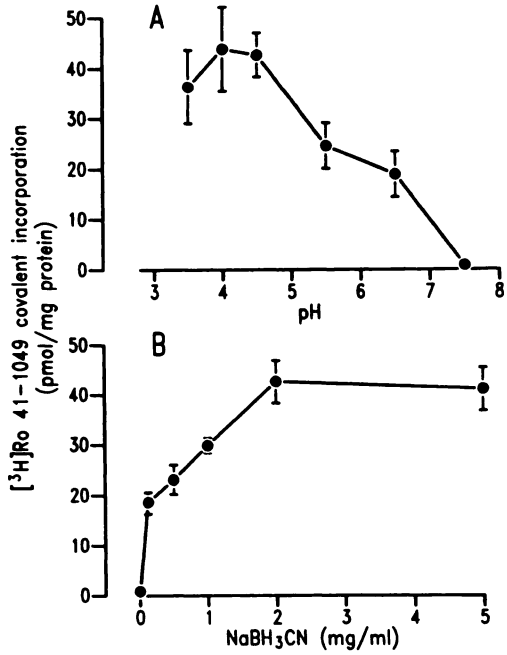

Fig. 6. Covalent irreversible incorporation of [^{3}H]Ro 41-1049 into human placenta membranes (previously incubated with 100 nM radioligand for 1 hr at 37°) after the addition of $NaBH_3CN$ (2 mg/ml) at different pH values (A) or of various amounts of $NaBH_3CN$ at pH 4.5 (B). Irreversibly bound radioactivity was separated from the reversibly bound as described in the test. *Points* are means ± standard errors of three experiments.

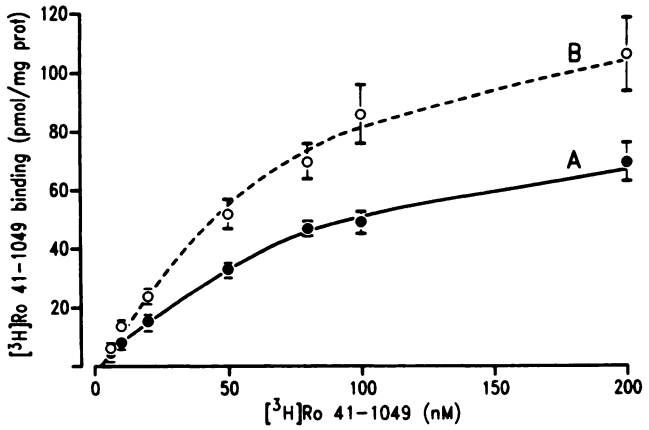

Fig. 7. A, Covalent irreversible incorporation of [^{3}H]Ro 41-1049 into human placenta membranes, at various ligand concentrations, after treatment with $NaBH_3CN$ (2 mg/ml, pH 4.5). B, Control curve of the [^{3}H] Ro 41-1049 reversible binding to human placenta membranes at various ligand concentrations (no $NaBH_3CN$ treatment). Points are means ± standard errors of three experiments.

TABLE 2

Kinetic parameters of [^{3}H]Ro 41-1049 binding to placenta membranes after affinity labeling ($NaBH_3CN$, pH 4.5) with 1 μM Ro 41-1049

Placenta membranes were incubated (1 hr at 37°) in the presence or in the absence (controls) of 1 μM unlabeled Ro 41-1049. $NaBH_3CN$ (2 mg/ml, final concentration) and 1% acetic acid (to pH 4.5) were then added where indicated and the samples were reincubated for 10 min at 20°. After centrifugation and rinsing, the membrane pellets were resuspended in Tris buffer and subsequently dialyzed for 5 hr at 37°. Membranes were finally resuspended in Tris buffer and assayed for [^{3}H]Ro 41-1049 binding as described in the text. Values are means ± standard errors of three experiments. The B_{max} percentage values are relative to the respective control.

	B_{max}		K_d
	pmol/mg of protein	*% of control*	*nM*
Control	88.5 ± 11.3	100	60.3 ± 10.5
Ro 41-1049	80.3 ± 7.0	91	52.2 ± 5.6
Control ($NaBH_3CN$, pH 4.5)	39.0 ± 1.1	100	45.9 ± 5.2
Ro 41-1049 ($NaBH_3CN$, pH 4.5)	18.1 ± 3.6	46	38.3 ± 7.0

41-1049, followed by dialysis to remove the reversibly bound inhibitor, the [^{3}H]Ro 41-1049 binding B_{max} was similar to the control value (Table 2). In contrast, when $NaBH_3CN$ was added (pH 4.5), the B_{max} value was greatly reduced. This indicates that an enzyme residue essential for its activity is modified after the affinity labeling reaction. It is noteworthy that the treatment with $NaBH_3CN$ and acetic acid caused by itself a marked irreversible inactivation of the binding activity (Table 2).

Characterization of the incorporation site of [^{3}H]Ro 41-1049 on MAO-A. After *in situ* limited proteolysis (V8 protease) of human placenta MAO-A affinity labeled with [^{3}H] Ro 41-1049 ($NaBH_3CN$, pH 4.5) or [^{3}H]pargyline, the pattern of the radioactive peptides produced was very similar for [^{3}H] Ro 41-1049- and [^{3}H]pargyline-labeled MAO-A (Fig. 8). In repeated experiments, no significant differences, even in the low molecular mass range, were observed in the size of the fragments labeled by the two radioligands.

To further investigate the site of incorporation of [^{3}H]Ro 41-1049, pure human placenta MAO-A previously affinity labeled with [^{3}H]Ro 41-1049 was also subjected to extensive proteolysis with trypsin. A single major radioactive band of 2.9 ± 0.1 kDa was seen after fluorography of the gel (Fig. 9). Trypsin proteolysis, under the same conditions, of pure MAO-A labeled with

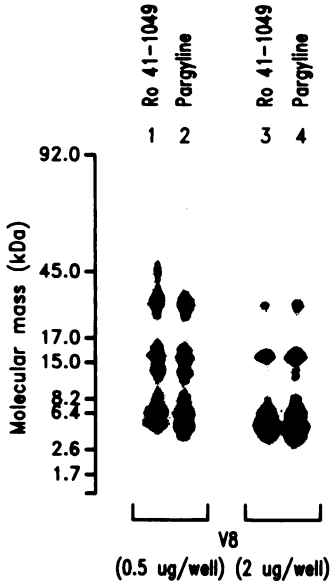

Fig. 8. Limited proteolysis (V8 protease) of human placenta MAO-A affinity labeled with 100 nM [^{3}H]Ro 41-1049 and 200 nM [^{3}H]pargyline (in the presence of 5 μM Ro 19-6327). Proteolysis was performed directly in the gel wells (30). The generated peptides were separated by SDS-PAGE (10% T, 3% C) (31). The average size of the two smaller labeled peptides was [^{3}H]Ro 41-1049-MAO-A, 6.8 ± 0.2 and 5.1 ± 0.2 kDa; [^{3}H]pargyline-MAO-A, 6.7 ± 0.2 and 4.9 ± 0.2 kDa (three experiments). The molecular mass (kDa) scale given in the *ordinate* was obtained by using phosphorylase B (92) and ovalbumin (45) as protein markers and myoglobin CNBr fragments as marker peptides (PMW calibration kit; Pharmacia), intact myoglobin (17), myoglobin I+II (15), myoglobin I (8.2), myoglobin II (6.4), myoglobin III (2.6), myoglobin 1–14 (1.7).

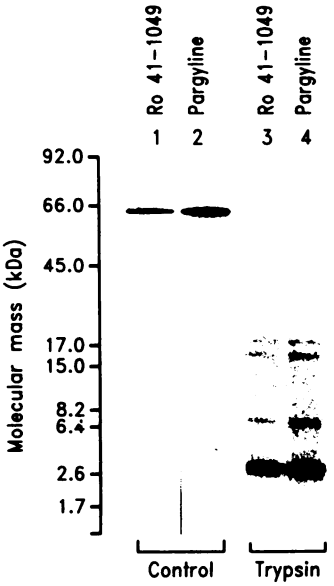

Fig. 9. Extensive trypsin proteolysis of pure human placenta MAO-A affinity labeled with [^{3}H]Ro 41-1049 and [^{3}H]pargyline. The tryptic fragments were separated by SDS-PAGE (16% total acrylamide concentration, 3% crosslinker concentration) (31). For details of the procedure, see Materials and Methods. The molecular mass (kDa) scale given in the *ordinate* was obtained using phosphorilase B (92), albumin (66), and ovalbumin (45) as protein markers and the myoglobin CNBr fragments (Pharmacia PMW calibration kit) as peptide markers (for molecular mass values, see legend to Fig. 8).

[^{3}H]pargyline also produced a major single fragment of identical apparent molecular mass.

Discussion

The results shown in the present study indicate that [^{3}H]Ro 41-1049 can be used as a reversible as well as an affinity labeling ligand for the characterization of MAO-A in binding experiments. In addition, our experiments provide evidence that [^{3}H] Ro 41-1049 binding sites correspond to the active sites of MAO-A.

In human brain frontal cortex, the amount of MAO-A, determined as [^{3}H]Ro 41-1049 B_{max}, was of the same order of magnitude as the values found by other authors who titrated MAO-A with clorgyline (37). In our opinion, however, [^{3}H] 41-1049 binding is a more direct method capable of yielding better estimates of MAO-A concentrations than enzyme titration with clorgyline, which has been found to give overestimated values due to drug nonspecific binding (38). In addition, [^{3}H]Ro 41-1049 binding experiments using rat brain membranes showed a B_{max} (6.6 ± 0.8 pmol/mg of protein) very similar to the B_{max} value previously found for [^{3}H]harmaline binding to similar membrane preparations (20). The density of [^{3}H]Ro 41-1049 binding sites was much higher in human placenta preparations than in human frontal cortex. It is worth mentioning that [^{3}H] Ro 16-6491 binding experiments showed that all our placenta preparations contained trace amounts of MAO-B (about 3–5% of MAO-A content). The presence of some MAO-B both in crude and in purified MAO-A preparations from human placenta has been also detected by other investigators using immunological methods (39). It is not clear yet, however, whether this MAO-B activity is intrinsic to placenta cells or whether it derives from extraplacenta elements, e.g., clotted platelets adhering to blood vessels.

The potencies of various drugs in competing for the [^{3}H]Ro 41-1049 binding closely paralleled their affinities for MAO-A, thus indicating that the ligand binding site corresponds to MAO-A and, precisely, to the enzyme active site.

Altogether, [^{3}H]Ro 41-1049 represents a new probe for binding studies of MAO-A in various tissues. Moreover, as already reported for the MAO-B ligand [^{3}H]Ro 19-6327 (40, 41), [^{3}H] Ro 41-1049 may serve in enzyme radioautographic studies on tissue slices, for the precise selective localization of MAO-A in discrete brain areas as well as in extracerebral organs.

It is noteworthy that, as reported previously for the MAO-B ligands, [^{3}H]Ro 16-6491 and [^{3}H]Ro 19-6327 (24, 25), [^{3}H]Ro 41-1049 behaves as substrate for the MAO-A isoenzyme. Thus, [^{3}H]Ro 41-1049, after its oxidation by MAO-A, appears to be activated into an intermediate that remains tightly bound to the enzyme through a not yet elucidated interaction (labile covalent and/or noncovalent linkage). It is important to note that Ro 41-8328, the aldehyde derivative of Ro 41-1049, in which the membrane-bound radioactivity is recovered after the extraction procedure, did not show any affinity for MAO-A. Due to the complex interaction of [^{3}H]Ro 41-1049 with MAO-A, all the kinetic constants obtained from [^{3}H]Ro 41-1049 binding have, thus, to be considered with caution because they are the result of a complex process, namely the transformation of the inhibitor into a second unknown species, which in turn interacts with the enzyme active site.

The same mechanistic considerations previously discussed for the two MAO-B ligands [^{3}H]Ro 16-6491 and [^{3}H]Ro 19-6327 (24–26) can be applied to the interaction of [^{3}H]Ro 41-1049 with MAO-A. A simplified scheme is proposed in Fig. 10. These inhibitors have the same operator part for MAO on their molecule, i.e., the 2-aminoethyl-carboxamide moiety, which is probably very important for the stabilization of the enzyme-inhibitor adduct (24, 26), whereas the aromatic "head" of the molecules appears to determine the binding selectivity toward the different recognition sites of the two MAO forms.

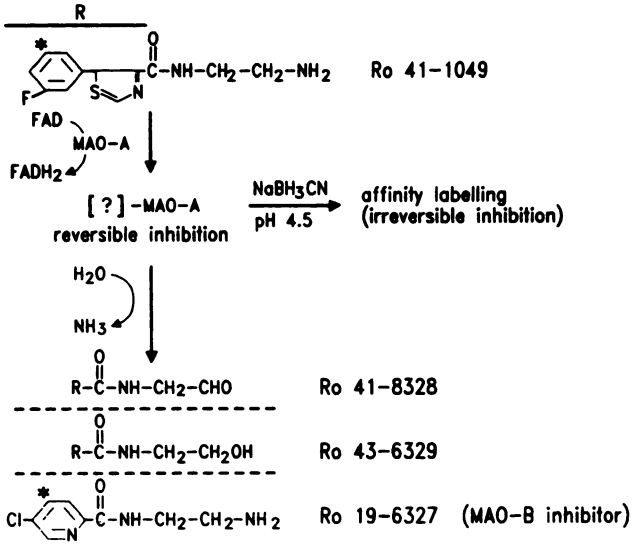

Fig. 10. Proposed scheme for the interaction of Ro 41-1049 with MAO-A.

Of interest, both for the elucidation of the interaction mechanism of these inhibitors and for the study of the structure of the MAO-A active site, is the reaction with $NaBH_3CN$ under acidic conditions, which converts the reversible interaction of [^{3}H]Ro 41-1049 into an irreversible one. The available data strongly suggest that the labeled polypeptide corresponds to a MAO-A subunit. To our knowledge, [^{3}H]Ro 41-1049 represents the only selective radioactive affinity label for MAO-A available so far. Furthermore, its selectivity for MAO-A appears to be higher than that of clorgyline, which loses its selectivity and inhibits also MAO-B at micromolar concentrations (4).

As calculated from the reversible and irreversible binding saturation curves (Fig. 7), about 70% of the bound ligand appeared irreversibly incorporated into the enzyme after the $NaBH_3CN$ reduction step. The optimal conditions for [^{3}H]Ro 41-1049 affinity labeling of MAO-A were identical to those previously found for the affinity labeling of MAO-B by [^{3}H]Ro 16-6491 (26) or [^{3}H]Ro 19-6327.[1] However, with the MAO-B inhibitors, only 50% radioactivity incorporation was normally achieved. The fact that we were unable to obtain 100% radioactivity incorporation is probably due to the poor stability of the ligand-enzyme complex observed at pH 4.5 (data not shown).

In order to localize the protein domain that is covalently linked to Ro 41-1049 after the $NaBH_3CN$ reduction, we compared the labeled peptide maps produced after both limited and extensive proteolysis of human placenta MAO-A that was affinity labeled with [^{3}H]Ro 41-1049 or with [^{3}H]pargyline, an irreversible MAO inhibitor that is known to form a stable adduct with the FAD coenzyme (42). The results suggest that

[1] Unpublished observations.

by the reduction step [^{3}H]Ro 41-1049 is incorporated into the same peptide fragment of the enzyme that also contains FAD. In addition, considering the predicted amino acid sequence of human placenta MAO-A (8), the apparent molecular mass of the labeled 2.9-kDa peptide produced after extensive trypsin proteolysis is in relatively good agreement with the size of the 26-amino acid FAD-peptide fragment (Leu 396–Arg 422) that would be generated by the complete trypsin digestion of MAO-A.

Because similar results were obtained after trypsin digestion of MAO-B labeled with [^{3}H]Ro 19-6327 or [^{3}H]pargyline,[1] Ro 41-1049 and the MAO-B inhibitors appears to label a domain on MAO-A and MAO-B, i.e., the FAD-peptide fragment, that is highly conserved in the two MAO forms (8, 43) and, most probably, not essential for substrate or inhibitor specificity.

We have previously postulated that a lysine residue might be involved in the covalent interaction of Ro 19-6327 with the enzyme (25). However, because no lysine appears to be present on the tryptic FAD-peptide fragment of either form of MAO, another enzyme residue is probably involved in the covalent interaction of these inhibitors with the respective MAO form.

The study of the spectral modification of FAD during the reversible and the irreversible inhibition, together with the identification of the enzyme moiety modified by the irreversible labeling, will greatly help in the understanding of the mechanism of action of this class of MAO inhibitors.

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