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ACCELERATED COMMUNICATION

Localization of the Imidazoline Binding Domain on Monoamine Oxidase B

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SUMMARY

Monoamine oxidase B (MAO-B) was recently identified as a member of the family of imidazoline binding proteins. To localize the imidazoline binding domain on MAO-B, we labeled the domain with the imidazoline photoaffinity adduct [^{125}I]2-(3-azido-4-iodophenoxy)methylimidazoline in rat and human liver and visualized labeled peptides by autoradiography/sodium dodecyl sulfate-polyacrylamide gel electrophoresis after CNBr cleavage of the labeled protein. Based on species-specific fragmentation patterns and immunoprecipitation of labeled peptides, the imidazoline binding domain was localized to residues K149 to M222 of human MAO-B. The imidazoline binding domain is encompassed within a region that influences substrate processing but is distinct from primary sites of interaction for the enzyme inhibitors pargyline and lazabemide (Ro 19–6327). Radioligand binding assays and photoaffinity labeling also indicated that the various classes of compounds did not cross-compete at the different enzyme domains. Identification of an imidazoline binding domain on MAO-B provides a new opportunity for the potential pharmacological development of imidazoline/guanidinium compounds and also presents additional avenues for structure/function analysis of the monoamine oxidase enzymes.

MAO-A and -B were recently identified as imidazoline binding proteins (1–3). Members of the family of imidazoline binding proteins recognize a number of pharmacologically active compounds that contain imidazoline/guanidinium moieties, including the α_1-AR agonist/α_2-AR antagonist cirazoline, the α_2-AR antagonist idazoxan, the α_2-AR agonist guanabenz, the ion-transport inhibitor amiloride and other structurally related ligands. Such molecules exhibit high affinity for membrane proteins (I1 and I2 imidazoline binding proteins) that are distinct from receptors for known hormones and these membrane proteins recognize endogenous bioactive substance(s) that mimic some of the pharmacological effects of these compounds (3, 4). Although these observations suggested the existence of a previously uncharacterized cell signaling system, the limited information on the identity and functionality of this family of imidazoline binding sites has hampered the full understanding of this system. I1 imidazoline binding proteins are localized to the plasma membrane, whereas the majority of I2 imidazoline binding proteins are found in mitochondrial membranes. Two major types of I2 imidazoline binding proteins are identical to the mitochondrial enzymes MAO-A and -B. The high affinity interaction of selected imidazoline/guanidinium compounds with MAO-A and -B suggests that some pharmacological effects of these compounds may involve altered levels of monoamine neurotransmitters via regulation of enzyme activity.

MAO-A (527 amino acids) and -B (520 amino acids) exhibit 73% amino acid identity and are localized to the outer membrane of the mitochondria, where the enzymes are estimated to constitute 0.5–1% of mitochondrial protein. MAO-A and -B catalyze the oxidative deamination of amine neurotransmitters such as dopamine and norepinephrine as well as various xenobiotics. The size of the substrate binding site has evolved to allow the MAO-A and -B enzymes to distinguish substrates and additional differences in their primary structures allow selective interaction with inhibitors. The MAO enzymes apparently possess several discrete motifs for interac-

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ABBREVIATIONS: MAO, monoamine oxidase; AR, adrenergic receptor; SDS, sodium dodecyl sulfate; AZIPI, 2-(3-azido-4-iodophenoxy) methylimidazoline; PAGE, polyacrylamide gel electrophoresis; PEA, phenylethylamine; BU-224, 2-(4,5-dihydroimidaz-2-yl-quinoline).

tion with a variety of compounds. The imidazoline binding domain on MAO-B seems to be distinct from the enzyme active site that recognizes the mechanism-based inhibitors, such as pargyline and deprenyl, and it is not equally accessible in liver and platelets, which suggests either tissue-specific regulation of the enzyme or a previously unidentified heterogeneity in MAO-B (2, 5). As an initial approach to these issues, we identified the enzyme domain covalently labeled with the imidazoline photoaffinity adduct [^{125}I]AZIPI.

Experimental Procedures

Materials. Carrier-free [^{125}I]sodium iodide was purchased from Du Pont-New England Nuclear (Boston, MA). [^{3}H]idazoxan (41 Ci/mmol) and low-molecular-mass range protein standards were purchased from Amersham (Arlington Heights, IL) and [^{3}H]Ro 19–6327 [*N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide HCl (lazabemide)] (17 Ci/mmol) was a kind gift from Dr. Grayson Richards (Hoffman-La Roche, Basel, Switzerland). Pargyline and Ro 16–6491 [*N*-(2-aminoethyl)-*p*-chlorobenzamide HCl] were provided by Research Biochemicals (Natick, MA) and cirazoline was a gift from Synthelabo (Paris, France). Protein A sepharose and CNBr were purchased from Sigma (St. Louis, MO). Kodak X-AR 5 film was purchased from Chesapeake X-Ray (Florence, SC). Acrylamide, bisacrylamide, SDS and prestained mid-range molecular mass protein standards were purchased from Bio-Rad (Hercules, CA). Ecoscint A scintillation fluid was purchased from National Diagnostics (Atlanta, GA).

Membrane preparation. Membranes were prepared from tissues as previously described (2). Frozen human and rat liver samples were homogenized in lysis buffer and filtered through two layers of gauze mesh. The lysate was centrifuged at 1,000 $\times$ *g* for 10 min at 4°. The pellet was discarded and the supernatant was centrifuged at 35,000 $\times$ *g* for 10 min at 4° to generate a crude membrane preparation. The resulting pellet was washed two times in membrane buffer (50 mM Tris, pH 7.5, 0.6 mM EDTA, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride), rapidly frozen and stored at −70° for up to 2 months. Protein was measured by the method of Lowry.

Photoaffinity labeling. The cirazoline derivative, 2-(3-amino-4-iodophenoxy) methylimidazoline (AMIPI) was synthesized, radioiodinated and converted to the photolabile azide ([^{125}I]AZIPI) for use as a photoaffinity adduct as described previously (6, 7). Membrane preparations were incubated in reduced light with 1–4 nM [^{125}I]AZIPI for 30 min at 24°, chilled on ice and diluted 10-fold with 4° membrane buffer containing 2 mM dithiothreitol immediately before photolyzing at 4° for 5 min in a Ray-O-Vac photolysis chamber (320 nm). Membrane preparations were pelleted in a microcentrifuge, solubilized in loading buffer (100 mM Tris·HCl, pH 6.8, containing 1% SDS, 50% glycerol, 25% β-mercaptoethanol and bromphenol blue) at 100° for 5 min and subjected to SDS-PAGE. Competing ligands were preincubated with the membrane preparations for 5–10 min at room temperature.

CNBr cleavage of photolabeled MAO-B. Photolabeled MAO-B was isolated by SDS-PAGE, the gel slice containing radioactivity was excised and the protein was digested to completion with CNBr (30 mg of CNBr in 70% formic acid for 48 hr at 24°). No further cleavage of the proteins was produced upon longer incubations with additional CNBr. An equal volume of water was added and the gel slice and solution was dried by rotary evaporation. For immunoprecipitation of CNBr fragments, the formic acid/CNBr mixture was removed from the gel slice after 48 hr and dried by rotary evaporation. The peptide fragments were resuspended in 50 mM Tris·HCl and the pH was adjusted (pH 7–9) with NaOH. The suspension was precleared with 10 μl (packed volume) of Sepharose A beads, incubated with a polyclonal antibody (1:100 final dilution) raised against a peptide corresponding to residues 203–217 of human MAO-B (8) for 12–16 hr at 4° and the antibody-antigen complexes were precipitated by addition of Sepharose A beads. The resulting pellet was washed in 50 mM Tris, pH 7.4, and solubilized in loading buffer at 100° for 10 min. The peptide fragments were separated on a 16.5% acrylamide gel using a Tricine buffer system (9) and radiolabeled peptides were detected by autoradiography. No fragmentation of the photoaffinity-labeled enzyme was observed after incubation with vehicle.

Radioligand binding. Membrane preparations were incubated with radioligands in the presence or absence of inhibitors for 20–30 min at 24° with shaking. Binding of [^{3}H]idazoxan was performed in the presence of 10 μM rauwolscine to block interaction with α_2-adrenergic receptors and nonspecific binding was determined in the presence of 10 μM cirazoline. Binding of [^{3}H]Ro 19–6327 was performed in the presence of 0.1 μM clorgyline to block interaction with MAO-A and nonspecific binding was determined in the presence of 100 μM Ro 16–6491. Membranes were collected by vacuum filtration on glass fiber filters (#32; Schleicher & Schuell, Keene, NH) and washed in 4 $\times$ 3 ml of 100 mM Tris pH 7.4 at 4°. Radioactivity retained on the filters was determined by liquid scintillation counting with approximately 60% efficiency.

Results and Discussion

The central location of MAO in the metabolism of biogenic amine neurotransmitters has generated substantial interest in terms of therapeutic management of diseases. A non-sense mutation in MAO-A is associated with sex-linked aggressive behavior and reduced expression of the enzyme may be a genetic link to mild retardation (10). Of particular note is the ability of MAO-B to potentially process "prodrugs" or toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (11). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine is oxidized by MAO-B to 1-methyl-4-phenylpyridinium^{+}, which induces a Parkinson's-like syndrome in humans and primates. MAO is actually a therapeutic target in the early management of Parkinson's disease, in which enzyme inhibition delays initiation of L-dopa administration. Related observations also suggest the involvement of the enzyme in various neurodegenerative diseases (e.g., amyotrophic lateral sclerosis, Alzheimer's disease) in which enzyme catalysis may liberate damaging oxygen free radicals. The imidazoline binding domain represents a potential regulatory domain on the enzyme. This site on the enzyme is not accessible in all tissues suggesting that imidazoline/guanidinium compounds selectively interact with a subpopulation of MAO (2).

The imidazoline photoaffinity adduct [^{125}I]AZIPI was used to covalently radiolabel MAO-B (2) and a strategy to identify the imidazoline binding domain on the enzyme was developed based on peptide mapping of the [^{125}I]AZIPI-labeled enzyme in different species. Both rat and human MAO-B were selectively photoaffinity-labeled with [^{125}I]AZIPI (Fig. 1, *left*) and saturation photolabeling of human[1] and rat MAO-B indicated that [^{125}]AZIPI is recognized by the imidazoline binding domains on the two enzymes with similar affinities (K_d ~2–3 nM) (8, 12). The primary structures of human and rat MAO-B are 88% identical (13, 14); however slight differences in the position of methionine residues in the proteins lead to discreet but readily identifiable differences in the CNBr fragmentation pattern. The predicted CNBr fragments from human and rat MAO-B are indicated in Table 1. If the photoprobe were incorporated into one of the larger peptide fragments exhibiting species variation in size, the subtle differences in the size of the digested peptides might allow identification of the imidazoline binding domain

[1] Raddatz, R. and S. M. Lanier, unpublished observations.

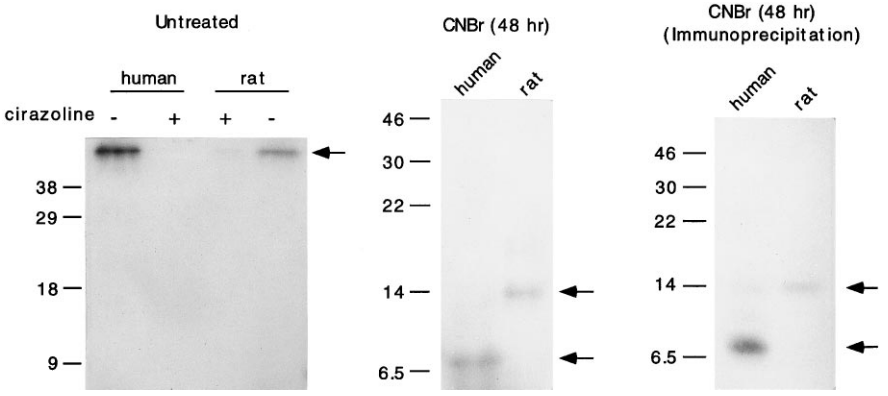

Fig. 1. Localization of the imidazoline binding domain on MAO-B. Autoradiographs of radiolabeled human and rat MAO-B and CNBr fragments of the proteins. Human and rat liver membranes (~500 μg of protein) were photoaffinity labeled with [^{125}I]AZIPI (4 nM) in the presence or absence of cirazoline (100 μM) and proteins were separated by SDS-PAGE. The photoaffinity-labeled enzyme was excised from the gel and incubated with vehicle (*left*) or with CNBr (*center*) as described in Experimental Procedures. In parallel experiments, the samples cleaved with CNBr were incubated with an antipeptide antibody (1:100 dilution) that recognizes the sequence between amino acids 203 and 217 of MAO-B and were processed for immunoprecipitation as described in Experimental Procedures. The intact proteins (*left*), cleaved peptides, (*center*), or immunoprecipitated material (*right*) were then separated by electrophoresis on denaturing 16.5% polyacrylamide gels using a tricine buffer system. Autoradiographs were obtained by exposing dried gels to films for 7–14 days. *Numbers to the left,* migration of midrange molecular mass standards ($M_r \times 10^{-3}$). *Arrows,* migration of the intact MAO-B proteins near the top of the separating gel (*left*) or of cleaved peptides with M_r ~7,500 for human and M_r ~13,200 for rat MAO-B (*center* and *right*). Results are representative of two or three experiments.

after SDS-PAGE. CNBr cleavage of both the human and rat enzyme yielded one major peptide that was labeled by the photoaffinity adduct (Fig. 1, *middle*). The M_r of the radiolabeled fragments generated from rat and human MAO-B was ~13,000 and ~7,500, respectively. Radiolabeled fragments of approximately this size would result from photoincorporation of [^{125}I]AZIPI into a residue(s) between K149-M222 of human and K149-M270 of rat MAO-B (Table 1 and Fig. 2). A polyclonal antibody generated against a peptide corresponding to residues 203–217 of human MAO-B was thus used to identify the radiolabeled fragment. This antibody immunoprecipitated the radiolabeled CNBr fragment from human and rat liver MAO-B (Fig. 1, *right*), confirming the site of localization determined by the relative migration of the labeled peptide in the two species. Approximately 50% of the labeled fragment from human MAO-B was immunoprecipitated, whereas the fragment from rat MAO-B was recognized with lower efficiency. The radiolabeled fragments were not immunoprecipitated after incubation with preimmune rabbit serum.

Thus the imidazoline photoaffinity adduct [^{125}I]AZIPI covalently incorporates into an amino acid(s) between K149

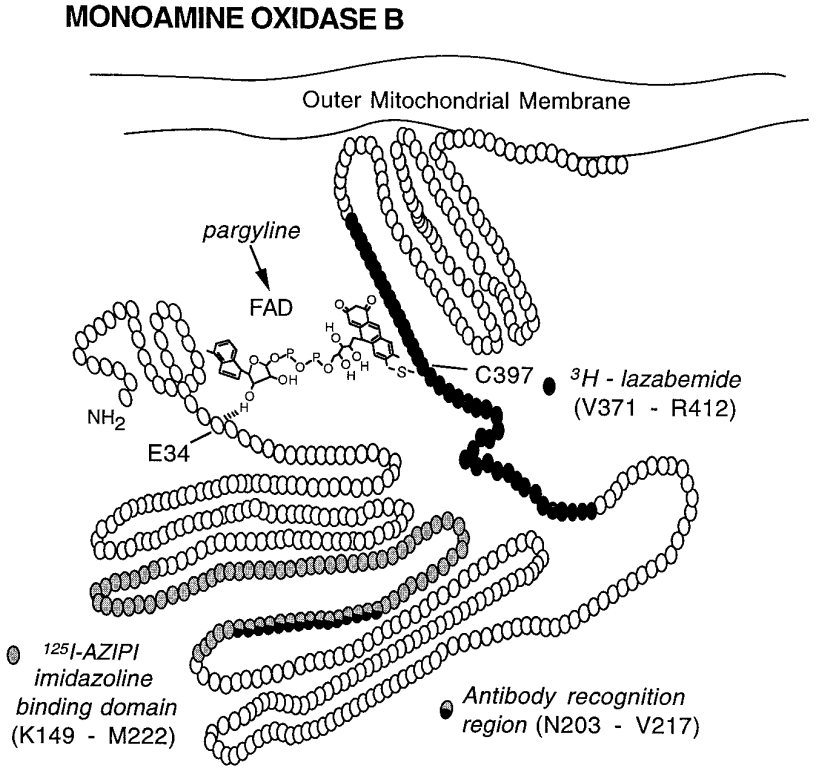

Fig. 2. Schematic representation of MAO- B. Human MAO-B consists of 520 amino acids (each represented by a *circle*). A dinucleotide recognition consensus site, including E34, is located near the amino terminus of the protein and the FAD is covalently attached to C397. Membrane association involves the carboxyl terminus of MAO-B. *Shaded circles,* the imidazoline binding domain interacting with [^{125}I]AZIPI (K149-M222); *half-filled circles,* the segment of the protein (residues 203–217) used to generate antipeptide antibodies. The acetylenic, irreversible enzyme inhibitors, such as pargyline, interact covalently with the FAD cofactor. Reversible inhibitors structurally related to Ro 19–6327 interact with a domain of the enzyme surrounding the FAD incorporation site (V371-R412, *filled circles*).

TABLE 1
Predicted CNBr fragments of rat and human MAO-B

CNBr cleavage of rat MAO-B[a]	Size		CNBr cleavage of human MAO-B[a]
	Rat	Human	
2–17	1,535	1,521	2–17
18–122	11,959	12,061	18–122
126–146	2,433	2,412	126–146
149–270	13,658	8,266	149–222
		3,802	223–254
271–298	316	1,193	271–280
		547	282–285
299–315	2,198	3,595	286–315
316–341	2,694	2,797	316–341
342–436	11,001	11,020	342–436
437–519	9,003	1,910	437–454
		7,215	455–520

[a] The numbers correspond to the amino acids in the peptide fragments generated by CNBr cleavage (13, 14).

and M222 of the enzyme. This domain is distinct from regions of the enzyme active site identified previously by various enzyme inhibitors and substrates (Fig. 2). Acetylenic irreversible MAO inhibitors such as pargyline, clorgyline and deprenyl incorporate into the covalently attached FAD cofactor, whereas the reversible mechanism-based inhibitor Ro 19–6327 interacts with a region of MAO-B that encompasses the FAD incorporation site toward the carboxyl terminus of the enzyme (15). Nevertheless, selected imidazolines and guanidiniums inhibit MAO-B (1, 16, 17). However, the concentrations of imidazoline/guanidinium ligands that generally inhibit MAO are 10–50-fold greater than the amount of ligand required to saturate the imidazoline binding domain in radioligand binding studies. The actual functional consequences of the interaction of such ligands with the subpopulation of MAO-B possessing an accessible imidazoline binding domain remain unknown.[2]

The relationship between the imidazoline binding domain and enzyme domains interacting with various enzyme inhibitors and/or substrates was further investigated by determining the ability of various compounds to cross-compete at the different enzyme domains. The MAO-B inhibitors [^{3}H]Ro 19–6327 and Ro 16–6491 are reversible, competitive inhibitors (18), whereas pargyline irreversibly inhibits enzyme activity. Photoaffinity labeling of the imidazoline binding domain on MAO-B with [^{125}I]AZIPI was not inhibited effectively by concentrations of pargyline or Ro 16–6491 that completely block binding of [^{3}H]Ro 19–6327 to the enzyme, which suggests that these compounds interact with distinct sites on the enzyme (Fig. 3A, B). Similarly, the imidazoline compounds BU224 and cirazoline did not inhibit binding of [^{3}H]Ro 19–6327 to MAO-B (Fig. 3B) at concentrations that completely block covalent labeling of (Fig. 3A) or radioligand binding to imidazoline binding proteins (1 μM or less). The imidazoline binding site is thus structurally distinct from sites interacting with known enzyme inhibitors. The effect of MAO substrates on the binding of [^{3}H]idazoxan was also examined (Table 2). Dopamine and tyramine serve as substrates for both enzyme isoforms, whereas serotonin and

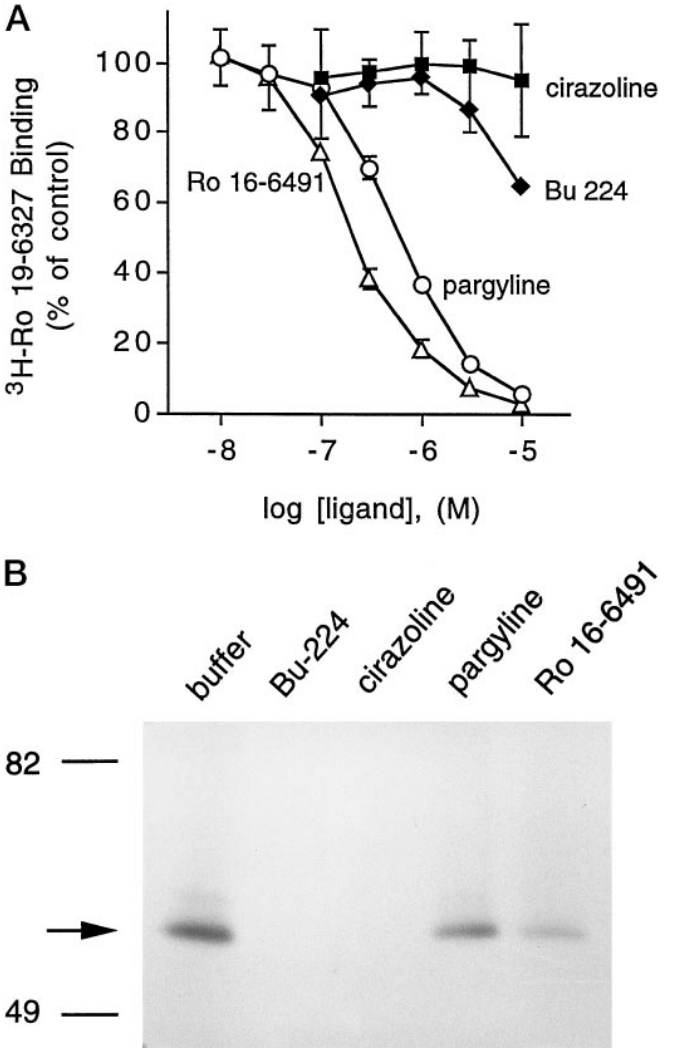

Fig. 3. Distinct binding sites on MAO-B for imidazoline compounds and competitive MAO inhibitors. A, Inhibition of [^{3}H]Ro19–6327 binding. Human liver membranes (100 μg of protein) were incubated with [^{3}H]Ro 19–6327 (12 nM) in the presence or absence of increasing concentrations of Ro 16–6491 (△), pargyline (○), BU224 (◆), or cirazoline (■) for 30 min at 24°. Total binding was 4570 ± 635 dpm and nonspecific binding was defined in the presence of 100 μM Ro 16–6491 (~5% of total binding). Results are expressed as percentage of specific binding in the absence of inhibitors and represent the mean ± standard deviation of two experiments. B, Inhibition of photoaffinity labeling. Human liver membranes (220 μg of protein) were photoaffinity labeled with [^{125}I]AZIPI (2 nM) in the presence or absence of BU224 (1 μM), cirazoline (1 μM), pargyline (10 μM), or Ro 16–6491 (10 μM). Autoradiographs were obtained by exposing dried gels to film for 3–5 days. The numbers to the left of the autoradiographs indicate the migration of midrange molecular mass standards ($M_r \times 10^{-3}$). *Arrow,* migration of proteins with M_r ~59,000. In some preparations, a labeled protein (M_r ~ 61,000) of lower intensity was observed. This labeled protein corresponds to MAO-A (2).

PEA are selectively metabolized by MAO-A and -B, respectively. The K_m exhibited by these substrates in MAO-B activity assays is ~3 μM for PEA, 200 μM for dopamine, 150 μM for tyramine and > 2 mM for serotonin (19). Binding of [^{3}H]Ro 19–6327 to MAO-B in liver membranes was displaced in a substrate selective manner (PEA > dopamine ~ tyramine > serotonin). Interestingly, binding of [^{3}H]idazoxan, a compound commonly used to identify imidazoline binding proteins, was also inhibited by enzyme substrates (PEA > tyra-

[2] Analysis of the relative densities of MAO-B and imidazoline binding sites indicated that in liver, the imidazoline binding domain was accessible on approximately one of every 20 molecules of MAO-B, whereas in platelet, the domain was accessible on only approximately one of every 200 molecules of the enzyme.

TABLE 2

Effect of enzyme substrates on radioligand binding to MAO-B in human liver membrane preparations

[^{3}H]Ro 19-6327 (30 nM) and [^{3}H]idazoxan (10 nM) binding were performed using 30 mg and 100 mg of human liver membrane protein, respectively. Results are expressed as the mean ± standard deviation of two experiments.

Enzyme substrate	Binding site	
	[^{3}H]Ro 196327	[^{3}H]Idazoxan
	K_i (μM)	
Phenylethylamine	11.7 ± 0.9	23.9 ± 0.7
Dopamine	218 ± 6.1	127 ± 26
Tyramine	293 ± 28	47.7 ± 12
Serotonin	>2 mM	257 ± 7.3

mine > dopamine > serotonin). Although the imidazoline compounds interact with a site on the enzyme distinct from MAO inhibitors, substrate binding and/or oxidation may influence the interaction of compounds with the imidazoline binding domain.

Functional studies using MAO-A and -B enzyme chimeras indicate that the region of the protein that encompasses the imidazoline binding domain plays an undefined role in determining substrate and inhibitor selectivity (20–22). The importance of this region of the enzyme relative to substrate processing is also indicated by the complete loss of activity observed after a mutation, which results in the substitution of Thr158 with Ala (15). As discussed above, the limited accessibility of the imidazoline binding domain on MAO-B in various tissues and the stoichiometric discordance between the density of I2 imidazoline binding proteins and MAO-B (2, 23) indicates the existence of a distinct "subpopulation" of enzyme molecules. Given the localization of the imidazoline binding domain in such a critical region of the enzyme, the tissue-specific accessibility of this enzyme domain is of particular interest. The identification of a distinct domain on MAO-B that exhibits high affinity for this class of pharmacologically active compounds may allow the development of novel therapeutic agents that target the enzyme in a cell-type selective manner.

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