Irrespective of the factors that might produce the effect, it does appear to be very difficult or impossible to directly cleave the cis form of P_2' substrates at enzyme concentrations that are realistically attainable, in agreement with our earlier suggestions (Lin & Brandts, 1983a).

In summary, the slow phase of proteolysis for substrates with proline at the P_2' position or at the active bond will probably always be rate-limited by the isomerization step. However, for substrates with proline at the P_2 position the cis form can be hydrolyzed, but at a much slower rate than the trans form so that the isomerization step may be rate-limiting at low enzyme concentration but not at high enzyme concentration. Consequently, certain precautions must be exercised in the interpretation of ISP data on such substrates. Fischer et al. (1984) have suggested that prolines as remote from the active bond as the P_4 and P_5 positions may exert an isomer-specific effect on the rate of hydrolysis even though there is no absolute preference for the trans form. Because of this, it appears likely that cleavage of active bonds in substrates with more than one proline residue located nearby may be very complicated.

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Revised Mechanism for Inactivation of Mitochondrial Monoamine Oxidase by *N*-Cyclopropylbenzylamine†

Michael L. Vazquez and Richard B. Silverman*

Departments of Chemistry and of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60201

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ABSTRACT: A mechanism previously proposed for inactivation of monoamine oxidase (MAO) by *N*-cyclopropylbenzylamine (N-CBA) [Silverman, R. B., & Hoffman, S. J. (1980) *J. Am. Chem. Soc. 102*, 884-886] is revised. Inactivation of MAO by N-[1-^{3}H]CBA results in incorporation of about 3 equiv of tritium into the enzyme and release of [^{3}H]acrolein. Treatment of inactivated enzyme with benzylamine, a reactivator for N-CBA-inactivated MAO, releases only 1 equiv of tritium as [^{3}H]acrolein concomitant with reactivation of the enzyme. Even after MAO is inactivated by N-[1-^{3}H]CBA, the reaction continues. At pH 7.2, a linear release of [^{3}H]acrolein is observed for 70 h, which produces 55 equiv of [^{3}H]acrolein while 2.3 equiv of tritium is incorporated into the enzyme. At pH 9, only 3.5 equiv of [^{3}H]acrolein is detected in solution after 96 h, but 40 equiv of tritium is incorporated into the enzyme, presumably as a result of greater ionization of protein nucleophiles at the higher pH. *N*-[1-^{3}H]Cyclopropyl-α-methylbenzylamine (N-CαMBA) produces the same adduct as N-CBA but gives only 1-1.35 equiv of tritium bound after inactivation of the enzyme. Denaturation of labeled enzyme results in reoxidation of the flavin without release of tritium, indicating attachment is not to the flavin but rather to an amino acid residue. Enzyme inactivated with N-[1-^{3}H]CαMBA is reactivated by benzylamine with the release of 1 equiv of [^{3}H]acrolein, which must have come from an adduct attached to an active site amino acid residue. About half of the tritium remains bound to the enzyme if the N-[1-^{3}H]CαMBA-inactivated enzyme is treated with a low concentration of sodium borohydride prior to benzylamine treatment. The adducts formed when N-CBA, N-CαMBA, and *N*-cyclopropyl-*N*-methylbenzylamine inactivate MAO appear to be identical. They have identical rates of reactivation by benzylamine at pH 7.2 and 9. These results suggest that N-CBA is oxidized by one electron to the amine radical cation followed by homolytic cyclopropyl ring cleavage and attachment to an active site radical, producing a 3-(amino acid residue)propanal adduct.

The compound *N*-cyclopropylbenzylamine (N-CBA)[1] was shown to be a mechanism-based (enzyme-activated) inactivator of mitochondrial monoamine oxidase (MAO, EC 1.4.3.4) (Silverman & Hoffman, 1980). A mechanism-based inactivator is an unreactive compound that is structurally related to the substrate or product of an enzyme and is converted by the enzyme to a species that inactivates the enzyme without prior release from the active site (Silverman & Hoffman, 1984; Walsh, 1984; Abeles & Maycock, 1976; Rando, 1974). The

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[1] Abbreviations: N-CBA, *N*-cyclopropylbenzylamine; MAO, monoamine oxidase; N-CαMBA, *N*-cyclopropyl-α-methylbenzylamine; N-CMBA, *N*-cyclopropyl-*N*-methylbenzylamine; 2,4-DNPH, 2,4-dinitrophenylhydrazine; Me_2SO, dimethyl sulfoxide; Tris-HCl, tris(hydroxymethyl)aminomethane.

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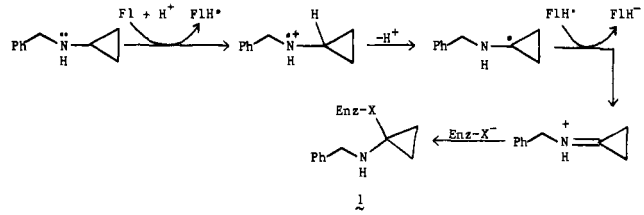
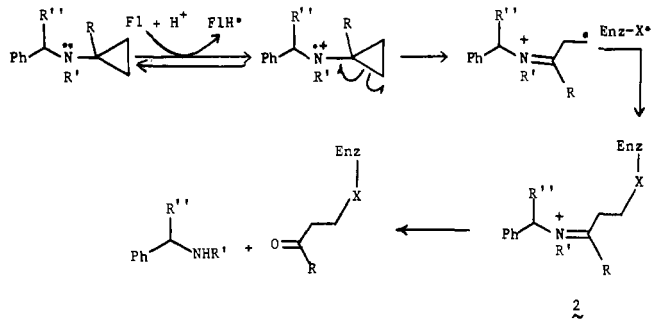
Scheme I: Previously Proposed Mechanism for MAO Inactivation by N-CBA[a]



[a] Fl represents the flavin cofactor; Enz-X⁻ is an enzyme-bound nucleophile.

Scheme II: Revised Mechanism for MAO Inactivation by N-CBA[a]



[a] Fl represents the flavin cofactor; Enz-X· is an enzyme-bound radical; R = H or ^3H; R′ = H or CH_3; R″ = H or CH_3.

mechanism that originally was proposed for inactivation of MAO by N-CBA (Silverman & Hoffman, 1980; Silverman et al., 1980) is shown in Scheme I. Recently, we have investigated the mechanisms for inactivation of MAO by various other cyclopropylamines and have concluded that *N*-(1-methylcyclopropyl)benzylamine (Silverman & Yamasaki, 1984), *trans*-2-phenylcyclopropylamine (Silverman, 1983), and 1-phenylcyclopropylamine (Silverman & Zieske, 1985) undergo a different reaction pathway than that proposed for N-CBA. The conclusion from those studies was that following one-electron transfer from the cyclopropylamine to the oxidized flavin cofactor, homolytic cyclopropyl ring opening occurs to give a reactive radical, which is captured by an active site radical. This also is the mechanism favored for cytochrome P-450 inactivation by cyclopropylamines and ethers (Guengerich et al., 1984). If this same mechanism were applied to N-CBA, the pathway shown in Scheme II would result. In this paper we show why the initial mechanism was proposed and present results that support the ring-opening mechanism (Scheme II).

Materials and Methods

Analytical Methods. These were described previously (Silverman & Yamasaki, 1984). Flash column chromatography was carried out by the method of Still et al. (1978).

Reagents. [7-^{14}C]Benzyl alcohol (9.5 mCi/mmol) was purchased from Pathfinder Laboratories, Inc. (St. Louis, MO). Sodium boro[^{3}H]hydride (400 mCi/mmol) was bought from ICN Chemical and Radioisotope Division (Irvine, CA). [7-^{14}C]Pargyline hydrochloride was a gift of Prof. Roy McCauley (Wayne State University). *N*-Cyclopropyl[*methylene*-^{14}C]-benzylamine (sp act. 1.08 × 10^6 dpm/μmol) was prepared from [*carbonyl*-^{14}C]benzaldehyde by the method of Silverman & Hoffman (1981). [7-^{14}C]Benzaldehyde was synthesized by the method of Ratcliffe & Rodehorst (1970) starting from [7-^{14}C]benzyl alcohol. N-CBA was prepared by the same method used to prepare the radioactive N-CBA. *N*-Methylcyclopropylamine was prepared as described by Grotjahn (1983). N-[1-^{3}H]CBA was prepared as described by Silverman & Hoffman (1981) (sp act. 8.17 × 10^6 dpm/μmol), and N-[1-^{3}H]Cα MBA (sp act. 4.77 × 10^6 dpm/μmol) was made previously by Silverman (1984). N-[1-^{3}H]CMBA was synthesized from N-[1-^{3}H]CBA by the method of Clarke et al. (1933).

All amines were converted to their hydrochloride salts and recrystallized from ethyl acetate. Tetrahydrofuran and ethyl ether were distilled under nitrogen from sodium with benzophenone ketyl as indicator. Hexane was dried over sulfuric acid and distilled from K_2CO_3. Methanol was distilled from magnesium. All other solvents were distilled before use. Methyl iodide was bought from Pfaltz and Bauer; sodium borohydride, dithionite, β-mercaptoethanol, 2,4-DNPH, Trisma base, and potassium pyrophosphate were purchased from Sigma Chemical Co.; all other reagents were obtained from Aldrich Chemical Co.

[7-^{3}H]Benzaldehyde. Benzaldehyde (51 μL, 0.5 mmol) was added all at once to a suspension of sodium borohydride (8.4 mg, 0.2 mmol) and sodium boro[^{3}H]hydride (10.5 mCi, 0.02 mmol) in methanol (1.0 mL) at 0 °C. After 15 min at 0 °C, the reaction mixture was allowed to warm to room temperature and to stir for an additional 45 min. The reaction was quenched by the addition of a few drops of 1.5 N HCl and then was diluted with water (4.0 mL). The mixture was extracted with ether (2 × 10 mL), which was washed with standard $NaHCO_3$ (1 × 5 mL) and saturated NaCl (1 × 5 mL). The ether layer was dried ($MgSO_4$) and carefully concentrated on a rotary evaporator. The crude [7-^{3}H]benzyl alcohol was oxidized to [7-^{3}H]benzaldehyde as previously reported (Ratcliff & Rodehorst, 1970). The specific activity of the [7-^{3}H]benzaldehyde (3.82 mCi/mmol) was calculated by recrystallization of its 2,4-dinitrophenylhydrazone derivative from ethanol–ethyl acetate (Shriner et al., 1964).

6-Hydroxy-4-thiahexanal 2,4-Dinitrophenylhydrazone. Acrolein (334 μL, 5.0 mmol) was added slowly to β-mercaptoethanol (386 μL, 5.5 mmol) containing 1 drop of 1.5 N HCl in an ice bath. After the addition was complete, the reaction was allowed to stir 15 min at room temperature and then was partitioned between ether and water (25 mL each). The layers were separated, and the aqueous layer was reextracted with ether (3 × 20 mL). The combined ether extracts were washed with saturated NaCl (2 × 10 mL) and dried ($MgSO_4$). The solvent was removed on a rotary evaporator leaving a clear oil, which was combined with 37 mL of a standard 2,4-DNPH solution (Shriner et al., 1964). The yellow precipitate was collected by suction filtration and washed with copious amounts of deionized water. The yield of product was 90%, which was pure by TLC (R_f 0.42, EtOAc; R_f 0.18, 1:1 EtOAc–*n*-hexane; R_f 0.32, 2% MeOH–CH_2Cl_2). A portion of the crude material (250 mg) was further purified by flash column chromatography (2.8 × 32 cm), eluting with ethyl acetate and collecting fractions of 5 mL. The product eluted in fractions 36–46, which were pooled, concentrated on a rotary evaporator, and dried in vacuo to give a yellow solid; mp 106–107 °C; NMR ([$^2H_6$]Me_2SO) δ 2.53–2.93 (br m, 6 H), 3.57 (dt, 2 H), 4.78 (t, 1 H), 7.83–8.90 (m, 4 H), and 11.40 (s, 1 H). Anal. Calcd for $C_{11}H_{14}N_4O_5S$: C, 42.03; H, 4.49; N, 17.82; S, 10.20. Found: C, 42.04; H, 4.66; N, 17.62; S, 10.06.

Enzyme and Assay. MAO was isolated from bovine liver mitochondria according to the method of Salach (1979). The specific activity of the purified enzyme varied from preparation to preparation and was in the range of 1.8–3.8 units/mg of protein. The enzyme was assayed by a modification of the method of Tabor et al. (1954) with 1 mM benzylamine hy-

drochloride in 20 mM potassium pyrophosphate, pH 9.0 buffer at 30 °C. All enzyme activities were assayed in air-saturated substrate solutions. A unit of activity is defined as the amount of enzyme required to convert 1 μmol of benzylamine to benzaldehyde per minute at 30 °C. Protein concentrations were determined by the method of Lowry et al. (1951).

General Procedure for MAO Inactivation. Concentrated MAO (25–45 mg/mL) was diluted 20-fold with 20 mM Tris-HCl or potassium pyrophosphate buffer, pH 9.0, containing 0.5–5.0 mM β-mercaptoethanol and then the inactivator was added to give a final concentration of 1 mM. The reaction mixture was incubated at 25 °C in the dark for 3 h and then was dialyzed at room temperature against 3 × 200 mL of 20 mM potassium phosphate, pH 7.2 buffer for at least 6 h. In all experiments, an enzyme control without inactivator and an inactivator control (for radioactive experiments) without enzyme were carried through the procedures simultaneously. All enzyme activities and measurements of radioactivity bound to enzyme were normalized by determination of protein concentrations. Active site determinations were carried out by [^{14}C]pargyline titration (Chuang et al., 1974).

Inactivation of MAO by N-[1-^{3}H]CBA and Identification of Acrolein as a Metabolite. MAO (200 μL, 2.8 mg/mL) was diluted with 5 mM N-[1-^{3}H]CBA in 20 mM potassium pyrophosphate, pH 9.0 (50 μL), and 30 mM Tris-HCl, pH 9.0 (250 μL). After 3.5 h at 25 °C, the reaction mixture was microdialyzed for 2.5 h against 20 mM potassium phosphate, pH 7.2 (3.0 mL), containing 10 mM β-mercaptoethanol. The microdialysis buffer was combined with 10 μL of 133 mM 2,4-DNPH reagent (Shriner et al., 1964), was allowed to incubate for 24 h at room temperature, and then was extracted with ethyl acetate (3 × 2 mL).

The enzyme solution after microdialysis was dialyzed against 3 × 200 mL of 20 mM potassium phosphate, pH 7.2 buffer, and an aliquot was counted for radioactivity. An aliquot (200 μL) of the dialyzed enzyme solution was added to an equal volume of 4 mM benzylamine hydrochloride in 20 mM Tris-HCl, pH 9.0 buffer containing 20 mM β-mercaptoethanol and was incubated at 25 °C for 3.5 h. The reactivated enzyme was microdialyzed, and the microdialysis buffer was treated with 2,4-DNPH reagent as above. After 12 h, it was extracted with 3 × 2 mL of ethyl acetate, and the combined extract was washed with 1 mL of water and evaporated. The residues obtained from both 2,4-DNPH reactions were analyzed by thin-layer chromatography on silica gel, eluting with either ethyl acetate or 1:1 *n*-hexane–ethyl acetate. The plates were dried and cut into 1-cm strips, and each strip was counted in 10 mL of scintillation fluid. The enzyme was dialyzed against 3 × 200 mL of 20 mM potassium phosphate, pH 7.2 buffer.

Time-Dependent Incorporation of Tritium into MAO and Release of Tritium into Solution upon Incubation of MAO with N-[1-^{3}H]CBA at pH 7.2 and 9. MAO (390 μM) in 50 mM potassium phosphate, pH 7.2 (30 μL), was incubated in the dark at 25 °C in 300 μL of 20 mM potassium phosphate, pH 7.2 buffer containing 1 mM N-[1-^{3}H]CBA. Periodically, aliquots (50 μL) were removed and microdialyzed against 20 mM potassium phosphate, pH 7.2 buffer (3.0 mL) for 1 h and then against 3 × 125 mL of the same buffer. Each of the microdialyzate buffers was applied to Dowex 50 X8 (200–400 mesh in H^+ form) (0.5 × 2 cm) equilibrated with deionized water. The columns were washed with water, and the radioactive non-amines were collected. Half of each fraction was counted for radioactivity, and to the other half was added 5 μL of a 133 mM solution of 2,4-DNPH. After incubation at room temperature for 24 h, the reaction mixtures were extracted with ethyl acetate (3 × 2 mL), the combined extracts were concentrated by rotary evaporation, and the residue was analyzed on two different supports (silica gel and silver nitrate treated silica gel) and two solvent systems (ethyl acetate and 1:1 ethyl acetate–*n*-hexane). The plates were cut into 1.0-cm strips, and each was counted in 10 mL of scintillation fluid. The dialyzed enzyme solution was tested for enzyme activity and then counted in 10 mL of scintillation fluid. All samples were normalized by determining the protein concentration. The same experiment was carried out in 20 mM potassium pyrophosphate, pH 9 buffer.

Inactivation of MAO by N-[1-^{3}H]CαMBA: Identification of Acrolein Released during Reactivation. MAO (800 μL, 1.875 mg/mL) in 20 mM Tris-HCl, pH 9.0 buffer was added to 200 μL of 10 mM N-[1-^{3}H]CαMBA. The samples were incubated at 25 °C in the dark for 2 h and then were dialyzed against 3 × 200 mL of 20 mM potassium phosphate, pH 7.2 buffer. A 400-μL aliquot was diluted with an equal volume of 4 mM benzylamine hydrochloride in 20 mM potassium phosphate, pH 7.2 buffer containing 10 mM β-mercaptoethanol. The reaction mixture was incubated in the dark at 25 °C for 3 h, and then 15 μL of a 133 mM solution of 2,4-DNPH was added. After incubation at room temperature for 14 h, the solution was extracted with 3 × 2 mL of ethyl acetate. The extracts were washed with 1 mL of water and concentrated in vacuo. The residue was analyzed by silica gel thin-layer chromatography, eluting with ethyl acetate. The plate was dried and cut into 1-cm strips, and each strip was counted in 10 mL of scintillation fluid.

Changes in the Flavin Spectrum upon Inactivation of MAO by N-CBA, N-CMBA, and N-CαMBA. Four aliquots of MAO (2.25 μM) in 495 μL of 20 mM potassium pyrophosphate, pH 9.0 buffer were prepared in a cuvette. An aliquot (5 μL) of the same buffer or buffer containing 1 mM N-CBA, N-CMBA, or N-CαMBA was added to the four samples. The change in absorbance relative to the control (no inactivator) was recorded between 350 and 750 nm at various times. Enzyme activity of each sample was determined by preparing identical samples as above and assaying as usual with time. After the enzyme was completely inactivated, each sample in the cuvettes was diluted with an equal volume of 8 M urea and allowed to stand in the dark for 15 min; then, the spectra were recorded relative to the control. To each of these samples was added 2 μL of 10% (w/v) sodium dithionite, and the spectra were again recorded.

Reactivation by Benzylamine at pH 7.2 and 9.0 of MAO Inactivated by N-CBA, N-CMBA, and N-CαMBA. MAO (8.7 μM) in 20 mM potassium pyrophosphate, pH 9.0 buffer (100 μL) was diluted with an equal volume of buffer containing 2 mM of either N-CBA, N-CMBA, or N-CαMBA. A control containing no inactivator also was prepared. All samples were allowed to incubate at 25 °C in the dark for 3 h and then were dialyzed against 2 × 200 mL of 20 mM potassium phosphate, pH 7.2 buffer. Each enzyme solution (100 μL) was diluted with an equal volume of 2 mM benzylamine in the same buffer, and at various times 20-μL aliquots were removed and diluted into 480 μL of assay buffer and the enzyme activity determined.

RESULTS

Inactivation of MAO by N-[1-^{3}H]CBA and Identification of Acrolein as a Metabolite. Analysis of the non-amine tritium products released during inactivation showed that 70% comigrated by TLC with that of the 2,4-dinitrophenylhydrazone of 6-hydroxy-4-thiahexanal, the Michael addition product of β-mercaptoethanol and acrolein. Another 11% comigrated

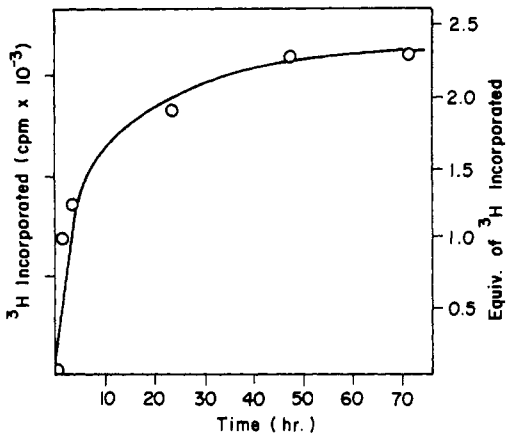


FIGURE 1: Incorporation of ^{3}H into MAO during inactivation with N-[1-^{3}H]CBA at pH 7.2. See Materials and Methods.

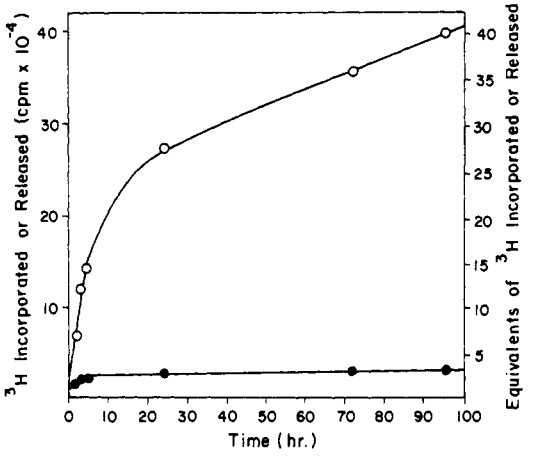


FIGURE 2: Time-dependent incorporation of ^{3}H into MAO during inactivation with N-[1-^{3}H]CBA (○) and release of [^{3}H]acrolein at pH 9 (●). See Materials and Methods.

with the 2,4-dinitrophenylhydrazone of acrolein. In all of our model reactions of β-mercaptoethanol with acrolein, the formation of what was believed to be a polymer of acrolein was observed. The remaining 19% of product radioactivity in this experiment comigrated with this unknown compound. The β-mercaptoethanol was added to the dialysis buffer to trap the acrolein and minimize polymer formation.

Under the conditions of this experiment, 3.11 equiv of tritium was incorporated into the enzyme after inactivation. Benzylamine treatment completely reactivated the enzyme and released only 0.95 equiv of radioactivity (2.16 equiv remained bound). Eight-six percent of the released radioactivity applied to the TLC plate comigrated with the 2,4-dinitrophenylhydrazone of 6-hydroxy-4-thiahexanal.

Time-Dependent Incorporation of Tritium into MAO and Release of [1-^{3}H]Acrolein into Solution upon Incubation of MAO with N-[1-^{3}H]CBA at pH 7.2 and 9.0. At pH 7.2, incorporation of tritium into MAO is rapid and linear for the first 6 h and amounts to slightly greater than 1 equiv of tritium per active site, at which point the enzyme has almost no activity relative to the enzyme control. A slow increase in incorporation of tritium into MAO proceeds over the next 68 h to give a final stoichiometry of 2.3 equiv of tritium (Figure 1). After 3 h, the enzyme had 5% activity and approximately 3 equiv of tritium, shown to be [1-^{3}H]acrolein as its 2,4-dinitrophenylhydrazone (R_f 0.65, EtOAc; R_F 0.58, 1:1 EtOAc–*n*-hexane; R_f 0.58, 2% MeOH–CH_2Cl_2), were generated. Further incubation resulted in a linear increase of [1-^{3}H]-acrolein generated over 70 h, after which time 55 equiv was produced.

At pH 9, inactivation of the enzyme was faster than at pH 7.2 with only 9% activity remaining after 1.5 h. At this time, 7 equiv of tritium was incorporated into MAO, and 1.7 equiv of tritiated non-amines were released. This trend continued through 96 h at which time 40 equiv of tritium was bound to the enzyme and 3.5 equiv of tritiated non-amines was released (Figure 2).

Inactivation of MAO by N-[1-^{3}H]CMBA and N-[1-^{3}H]-CαMBA. Incubation of MAO with N-[1-^{3}H]CMBA at pH 9 for 5 h resulted in the incorporation of 2.2 equiv of tritium. Inactivation by N-[1-^{3}H]CαMBA resulted in the incorporation of 1.07–1.35 equiv of tritium (several different experiments) per active site.[2] Treatment of labeled enzyme with benzylamine resulted in the return of 100% enzyme activity and the release of 1.0 equiv of tritium. Chromatography of the extracted solution after reactivation gave two bands of radioactivity; one, which was equal to 0.34 equiv of released radioactivity, comigrated with carrier 2,4-dinitrophenylhydrazone of 6-hydroxy-4-thiahexanal and the other (0.1 equiv of tritium) was not identified. The rest of the radioactivity remained in the aqueous layer and may be ^3H_2O from oxidation of the aldehyde.

Attachment of N-[1-^{3}H]CαMBA Is to an Amino Acid Residue. When N-[1-^{3}H]CαMBA-inactivated MAO was denatured in 8 M urea for 12 h, 91% of the tritium remained bound to the enzyme. Treatment with 4% trichloroacetic acid and 2% NaOH resulted in the retention of 78% and 59% of the radioactivity, respectively.

Changes in the Flavin Spectrum upon Inactivation of MAO by N-CBA, N-CMBA, and N-CαMBA. Inactivation of MAO by these three amines resulted in concomitant change in the optical spectrum from that of oxidized flavin to reduced flavin. Denaturation resulted in rapid and complete reoxidation of the flavin to that of the control. Further treatment with dithionite converted all of the spectra back to that of reduced flavin.

Reactivation by Benzylamine at pH 7.2 and 9.0 of MAO Inactivated by N-CBA, N-CMBA, and N-CαMBA. At pH 7.2, the rates for reactivation by benzylamine of MAO inactivated by N-CBA, N-CMBA, and N-CαMBA were identical and pseudo first order, having a $t_{1/2}$ = 74 min. At pH 9, the $t_{1/2}$ value for each was 26 min.

Discussion

When N-CBA was originally studied as an inactivator of MAO (Silverman & Hoffman, 1980), one false assumption resulted in misleading conclusions about its mechanism of inactivation. Inactivation of MAO with N-[1-^{3}H]CBA produced a time-dependent release of non-amine tritium [tritium not retarded on Dowex 50 (H^+)]; after complete enzyme inactivation, 0.7 equiv of non-amine tritium had been released per active site (Silverman & Hoffman, 1980). At the time that this experiment was carried out, the notion of a one-electron mechanism was not yet in mind. Consequently, it was assumed that this released non-amine tritium was ^3H_2O, resulting from removal of the tritium as shown in Scheme I. This experiment was repeated in the study described here, and similar results were obtained (Figure 3). However, the tritiated non-amine released was identified as acrolein by TLC of its 2,4-dinitrophenylhydrazone. Concomitant with release of [^{3}H]acrolein during inactivation was an incorporation of

[2] Incubation for 26 h resulted in incorporation of 3.22 equiv of radioactivity.

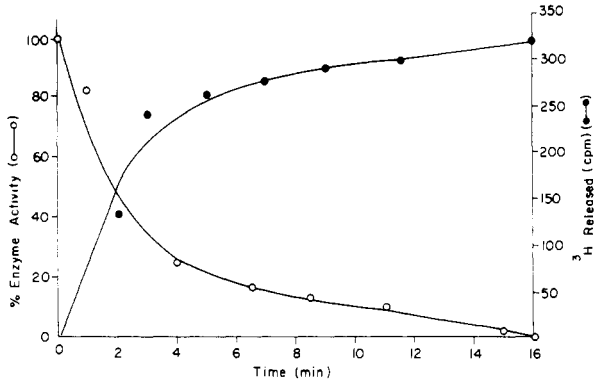


FIGURE 3: Release of [^{3}H]acrolein (●) concomitant with inactivation of MAO by N-[1-^{3}H]CBA (○).

tritium into the enzyme, and the amounts of each were pH-dependent. At pH 7.2, when the enzyme was 95% inactivated and approximately 1 equiv of tritium was incorporated (Figure 1), 3 equiv of [^{3}H]acrolein was isolated from solution. At pH 9, a much faster reaction ensued, in agreement with the higher activity of MAO at pH 9 than at pH 7.2, and when 91% of the enzyme was inactivated, 7 equiv of tritium was incorporated into the enzyme and only 1.7 equiv of [^{3}H]acrolein was free in solution (Figure 2). The higher incorporation of radioactivity into MAO at higher pH is consistent with the higher ionization of nucleophilic groups, especially cysteine, tyrosine, and lysine, which could undergo Michael additions with liberated [^{3}H]acrolein. This also accounts for the lower amount of [^{3}H]acrolein free in solution at the higher pH value. Surprisingly, when MAO was allowed to incubate with N-[1-^{3}H]CBA at pH 7.2 and 9 for a prolonged time following >95% inactivation of the enzyme, a linear time-dependent increase of [^{3}H]acrolein was isolated from solution at pH 7.2 and a linear time-dependent incorporation of radioactivity into the enzyme at pH 9 (Figure 2) was observed. We suspected that the continued generation of [^{3}H]acrolein following inactivation of the enzyme resulted from reactivation and further inactivation of the inactivated enzyme by N-CBA. Primary and secondary amines were shown to produce reactivation of N-CBA-inactivated MAO with concomitant release of acrolein (Yamasaki & Silverman, 1985). Since N-CBA is itself a secondary amine, it, too, could release [^{3}H]acrolein. The k_{inact} at saturation for N-CBA is 461 times greater than the k_{react} of saturation for *N*-methylbenzylamine, a model secondary amine for N-CBA (Yamasaki & Silverman, 1985); therefore, inactivation would occur much more rapidly than reactivation, and the enzyme would appear to have no activity throughout this process.

Only 1 equiv of the 3.1 equiv of tritium from N-[1-^{3}H]CBA which was incorporated into MAO during inactivation was released into solution following treatment with the known reactivator benzylamine (Silverman & Hoffman, 1980; Yamasaki & Silverman, 1985). Since full reactivation of the enzyme occurred with this released radioactivity, it may be inferred that 1 equiv of inactivator was bound to the active site, whereas the other 2 equiv were peripherally attached. The tritium released was identified as acrolein by conversion to the 2,4-dinitrophenylhydrazone of its β-mercaptoethanol Michael adduct (6-hydroxy-4-thiahexanal). However, because of the much greater than 1 equiv of tritium incorporated into MAO with N-[1-^{3}H]CBA, these results were not as satisfying as we desired. N-[1-^{3}H]CMBA was an improvement but still resulted in incorporation of more than 2 equiv of radioactivity into the enzyme. In a study on the effect of α-methylation of N-CBA on the partition ratio for inactivation of MAO, it was observed that N-[1-^{3}H]CαMBA inactivated MAO and resulted in the incorporation of only 1.20 equiv of radioactivity (Silverman, 1984). Since, as shown in Scheme II, the adducts formed from N-CBA, N-CMBA, or N-CαMBA inactivation of MAO would be identical after imine hydrolysis, we investigated the reaction of N-CαMBA as a model for N-CBA. Two experiments were carried out to determine if this hypothesis was reasonable. MAO inactivated by N-CBA, N-CMBA, and N-CαMBA was shown to be reactivated by benzylamine at identical rates. Furthermore, inactivation of MAO by these three compounds exhibited identical changes in the flavin spectrum before and after urea treatment. Both of these experiments suggest similar, if not identical, adducts. With N-[1-^{3}H]CαMBA in place of N-[1-^{3}H]CBA, only 1.07–1.35 equiv of tritium was initially incorporated. Treatment with benzylamine then resulted in the release of 1 equiv of radioactivity (identified as acrolein) concomitant with reactivation of the enzyme. This result, then, confirmed that the adduct attached to the enzyme active site was released as acrolein when treated with benzylamine.

Up to this point no mention has been made regarding the identity of the active site group to which N-CBA becomes attached. When N-[1-^{3}H]CαMBA- and N-[1-^{3}H]CBA-inactivated MAO were denatured, essentially no tritium was released from the enzyme. However, under identical conditions, the flavin became completely reoxidized. This suggests that N-CαMBA and N-CBA are not attached to the flavin coenzyme but, rather, to an active site amino acid residue. As was pointed out previously (Silverman, 1983; Silverman & Zieske, 1985), one-electron reduction of the flavin to a semiquinone may be followed by hydrogen atom abstraction of a nearby amino acid residue, resulting in an equilibrium mixture of flavin radical and amino acid radical. It is known that the amino acid adjacent to the cysteine residue to which the flavin is attached is tyrosine (Walker et al., 1971) and that there is at least one other active site cysteine residue (Singer & Barron, 1945). Both cysteine and tyrosine would be potential hydrogen atom donors. 1-Phenylcyclopropylamine was shown to become attached to a cysteine residue.[3] Proximity may determine whether attachment is to the flavin radical or the amino acid radical.

One difference that was observed in this study (data not shown) and the other N-CαMBA study (Silverman, 1984) compared with the original work (Silverman & Hoffman, 1980) is the stoichiometry of inactivating MAO with N-C[*phenyl*-^{14}C]BA. In the former two cases, it was found that when either N-CBA or N-CαMBA was labeled in the benzyl group, little radioactivity remained bound to MAO after inactivation and dialysis. However, in the latter case 1.1–1.4 equiv of N-C[*phenyl*-^{14}C]BA remained bound per active site after dialysis. At the time of the original work (Silverman & Hoffman, 1980), the MAO used for the study was purified from porcine liver mitochondria by the method of Oreland (1971).[4] Since that time, we have been using the method of Salach (1979) to purify MAO from both porcine and bovine liver mitochondria. In our work with *N*-(1-methylcyclopropyl)benzylamine (Silverman & Yamasaki, 1984), we showed that there was no difference in the results obtained with porcine or bovine liver MAO prepared by the Salach procedure. We do not know why the benzyl group radioactivity remained bound to MAO in the earlier case and not in the more recent work. According to the mechanism shown

[3] Silverman and Zieske, submitted for publication.

[4] The enzyme used in the earlier study was generously donated by Professor L. Oreland (University of Umeå).

in Scheme II, the Schiff base of the adduct in the earlier work apparently was protected from hydrolysis during dialysis, apparently as a result of the method of enzyme purification.

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Mechanism for Reactivation of *N*-Cyclopropylbenzylamine-Inactivated Monoamine Oxidase by Amines†

R. Bryan Yamasaki‡ and Richard B. Silverman*

Departments of Chemistry and of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60201

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ABSTRACT: The effect of 18 different amines, two mercaptans, and two alcohols on the reactivation of *N*-cyclopropylbenzylamine- (N-CBA-) inactivated bovine liver monoamine oxidase (MAO) is described. All of the compounds that reactivate the enzyme produce a time-dependent pseudo-first-order return of enzyme activity and exhibit saturation kinetics. There is no direct correlation between the ability of a compound to serve as a substrate for native MAO and its ability to reactivate N-CBA-inactivated MAO. Amines containing an aromatic moiety, in general, are better reactivators than the aliphatic amines. The amine must be primary or secondary in order for reactivation to occur. The distance between the aromatic portion and the amino group is critical to the reactivation properties of the compound. The mercaptans and alcohols do not reactivate N-CBA-inactivated MAO, nor do they interfere with the reactivation reaction by benzylamine. Three mechanisms for the reactivation reaction are considered. One involves initial Schiff base formation with the active site adduct produced by N-CBA inactivation of MAO followed by base-catalyzed β-elimination to the imine of acrolein. The second mechanism is the same as the first except no prior Schiff base formation is invoked. The third mechanism is an S_N2 displacement by the amine of the active site amino acid residue attached to the adduct. Experiments are carried out to exclude the S_N2 mechanism. The results of the reactivation experiments favor the Shiff base mechanism.

Mitochondrial monoamine oxidase (MAO,[1] EC 1.4.3.4), an enzyme containing a covalently bound FAD cofactor, catalyzes the oxidative deamination of biogenic amines. It has been shown in recent years that *N*-cyclopropyl-*N*-arylalkylamines are mechanism-based inactivators of MAO (Silverman et al., 1980; Silverman & Hoffman, 1980, 1981a; Silverman & Yamasaki, 1984; Silverman, 1984; Winn et al., 1975;

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‡ Present address: Native Plants, Inc., Plant Resources Institute, Salt Lake City, UT 84108.

[1] Abbreviations: MAO, monoamine oxidase; N-CBA, *N*-cyclopropylbenzylamine; N-[1-^{3}H]CBA, *N*-[1-^{3}H]cyclopropylbenzylamine; N-CαMBA, *N*-cyclopropyl-α-methylbenzylamine; N-CMBA, *N*-cyclopropyl-*N*-methylbenzylamine; FAD, flavin adenine dinucleotide.

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