Mechanism of Inactivation of Monoamine Oxidase by trans-2-Phenylcyclopropylamine and the Structure of the Enzyme-Inactivator Adduct*

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Mitochondrial monoamine oxidase was inactivated with 2-[2-14C]phenylcyclopropylamine, diazylated, and treated with acidic 2,4-dinitrophenylhydrazine. Contrary to the report of Paech et al. (Paech, C., Salach, J. I., and Singer, T. P. (1980) J. Biol. Chem. 255, 2700–2704), the 2,4-dinitrophenylhydrazone obtained was not that of 2-phenylecyclopropenone, but rather of cinnamaldehyde. Furthermore, denaturation of the labeled enzyme in the presence of sodium borohydride resulted in retention of 5.6 times more radioactivity than in its absence. Based on these results, a mechanism of inactivation of monoamine oxidase by 2-phenylcyclopropylamine and the structure of the enzyme-inactivator adduct are proposed.

2-PCPA,1 (1), an inhibitor of mitochondrial monoamine oxidase (EC 1.4.3.4) which is an enzyme containing covalently bound FAD, was introduced clinically as an antidepressant agent in 1961 (1, 2). In 1980, Paech et al. (3, 4) reported that 2-PCPA was a mechanism-based inhibitor of monoamine oxidase. A mechanism-based inhibitor is an unreactive compound which is converted by an enzyme into another species which, without prior release from the active site, inhibits the enzyme (5–8). The mechanism proposed (3, 4) for 2-PCPA inactivation of monoamine oxidase is shown in Scheme I. The structure of the enzyme adduct shown in Scheme I was suggested as a result of their proposed isolation of the 2,4-DNP of 2-phenylcyclopropenone upon acid denaturation of the inactivated enzyme in the presence of 2,4-DNPH. However, no structure proof was provided for the compound isolated. Cyclopropanones are exceedingly reactive species (9, 10). Cyclopropanone itself has been prepared and characterized by its IR spectrum only in the absence of nucleophiles and oxygen (11); however, phenylecyclopropanone, to the best of my knowledge, has never been isolated. Because of the instability of cyclopropanones and the absence of any known imine-like derivatives of 2-phenycyclopropanone, the reaction of acidic 2,4-DNPH with the adduct produced after inactivation of monoamine oxidase by 2-PCPA was reinvestigated. The product of this reaction was determined to be the 2,4-DNP of cinnamaldehyde, not of 2-phenylecyclopropanone as reported previously (3, 4).

**SCHEME 1.** Singer mechanism of inactivation of monoamine oxidase by 2-PCPA.

Materials and Methods—trans-2-PCPA hydrochloride was purchased from Sigma. 2-[2-14C]PCPA-H2SO4 (specific activity 1.6 × 106 dpm/μmol) was a generous gift of Dr. Carl Kaiser (Smith Kline and French Laboratories); [14C]pargyline-HCl (specific activity 3.06 × 106 dpm/μmol) was kindly donated by Dr. Roy McCauley (Wayne State University). Cinnamaldehyde (Aldrich) was converted to its 2,4-DNP by the method of Shriner et al. (12) and recrystallized from ethanol/ethyl acetate. TLC was carried out on Silica Gel 60 without fluorescence indicator (E. Merck). Research Products International 3a70B scintillation fluid was used for all radioactive counting.

Mitochondrial monoamine oxidase was isolated from beef liver by the method of Salach (13) and had a specific activity of 3.1–3.5 units/mg where 1 unit of activity corresponds to the oxidation of 1 μmol of benzylamine/min at 30 °C. The enzyme was assayed by the method of Taber et al. (14).

Release of Cinnamaldehyde from 2-[2-14C]PCPA—Inactivated Monoamine Oxidase—To 675 μl of the above inactivated enzyme solution was added 1350 μl of 10 mM 2,4-DNPH in 6.6% (w/v) perchloric acid. After standing at room temperature for 18 h, it was extracted with ethyl acetate (1.25 mL, 3 × 0.6 mL). The ethyl acetate extract, which contained 89% of the radioactivity previously bound to the enzyme, was divided into three equal portions. The solvent in each was evaporated with a stream of argon. To one portion was added 20 μl of ethyl acetate containing 1 μg each of cinnamic acid and the 2,4-DNP of cinnamaldehyde, and HPLC was carried out on a Beckman (Altek) Model 330 HPLC using an Altex ultraspHERE ODS S μ C18 silica gel column and methanol/water (8:2) as eluent at a 1.0 ml/min flow rate (T1/2 = 3.6 min for cinnamic acid; T1/2 = 13.3 and 14.0 min for presumably the two geometric isomers of the 2,4-DNP of cinnamaldehyde). Fractions of 1.0 ml were collected, diluted with 10 ml of scintillation fluid, and counted. Each of the other two portions of the ethyl acetate extract was dissolved in 50 μl of ethyl acetate and streaked along 6 cm of a silica gel TLC plate (10 × 20 cm) using cinnamic acid, cinnamaldehyde, 2,4-DNPH, and the 2,4-DNP of cinnamaldehyde as standards. The plates were developed with ethyl acetate containing 1 pg each of cinnamic acid and the 2,4-DNP of cinnamaldehyde. Fractions of 1.0 ml were collected, diluted with 10 ml of scintillation fluid, and counted. Each of the other two portions of the ethyl acetate extract was dissolved in 50 μl of ethyl acetate and streaked along 6 cm of a silica gel TLC plate (10 × 20 cm) using cinnamic acid, cinnamaldehyde, 2,4-DNPH, and the 2,4-DNP of cinnamaldehyde as standards.

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The abbreviations used are: 2-PCPA, trans-2-phenylcyclopropylamine; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 2,4-DNP, 2,4-dinitrophenylhydrazine; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.

1. The experimental conditions used were mimicked from those reported previously (3, 4).
cinnamaldehyde for reference. One plate was chromatographed in benzene:methanol (8:2) \((R_f = 0.96\) for cinnamaldehyde; \(R_f = 0.69\) for cinnamate); and the other in 3% methanol in chloroform \((R_f = 0.19\) for cinnamaldehyde; \(R_f = 0.73\) for cinnamate). One-cm strips were cut out and counted in 10 ml of scintillation fluid.

**Effect of Sodium Borohydride on the Stability of the Enzyme Adduct**—2-[2-\(^{14}\)C]PCPA was added to beef liver monoamine oxidase (8.7 \(\mu\)M) to give a final concentration of inactivator of 150 \(\mu\)M. After 1 h at 30°C, the solution was dialyzed at room temperature against 2 X 500 ml of 50 mM HEPES, pH 7.2, (14,800 dpm bound). The labeled enzyme was divided into two equal portions. To one half was added a solution of sodium borohydride (0.2 mg) in 4 \(\mu\)l of buffer, to the other, 4 \(\mu\)l of buffer. After 2 h at 25°C, each was cooled in ice, and to the NaBH₄-treated half was added 1.83 ml of cold 95% ethanol (293 mg). The product was taken up in alcohol (1 ml) and added dropwise to a stirred solution of 2,4-DNPH (72 mg, 0.36 mmol) in 9 ml of 10% perchloric acid. The orange-red precipitate was recrystallized twice from ethyl acetate, giving red-orange crystals (26 mg).

**Chemical Conversion of 2-PCPA to the 2,4-DNP of Cinnamaldehyde**—These procedures were kindly provided by Dr. Christian Paech (South Dakota State University) and were repeated exactly as described. 2-PCPA-HCl (1.0 g, 5.9 mmol) was suspended in 6 N HCl (3 ml) and stirred in an ice bath while a solution of sodium nitrite (414 mg, 6.0 mmol) in 2.4 ml of water was added dropwise. After 5 min, a few granules of urea were added and the mixture was heated in a steam bath for 5 min, cooled, and extracted with ether (2 × 20 ml). The combined ether extracts were washed with saturated NaHCO₃, water, and brine, dried (MgSO₄), and evaporated to a yellow liquid (293 mg). The product was redissolved in ether (1.5 ml) and added dropwise to a stirred solution of 2,4-DNPH (72 mg, 0.36 mmol) in 9 ml of 10% perchloric acid. The orange-red precipitate was recrystallized twice from ethyl acetate, giving red-orange crystals (26 mg).

**RESULTS**

**Inactivation of Monoamine Oxidase by 2-[2-\(^{14}\)C]PCPA**—Relative to active site labeling of monoamine oxidase by \(^{14}\)C pargyline (set equal to 1.0), 1.08 mol of 2-[2-\(^{14}\)C]PCPA remained bound per mol of monoamine oxidase (in agreement with Paech et al. (3, 4)).

**Release of Cinnamaldehyde from 2-[2-\(^{14}\)C]PCPA-inactivated Monoamine Oxidase**—HPLC of the labeled enzyme treated with acidic 2,4-DNPH resulted in recovery of 47% of the total counts in the 2,4-DNP of cinnamaldehyde fractions (Fig. 1). Silica gel TLC showed a yellow band which co-migrated with the 2,4-DNP of cinnamaldehyde and which contained 47 and 45% (for the two different solvent systems) of the total radioactivity applied (Fig. 2, A and B respectively). Less than 2% of the radioactivity migrated in the 2,4-DNP of cinnamaldehyde fraction of a nonenzyme control reaction. Almost all of the radioactivity in the nonenzyme control reaction migrated with fractions 3 and 4 in Fig. 2A and fraction 1 in Fig. 2B.

**Effect of Sodium Borohydride on the Stability of the Enzyme Adduct**—Sodium borohydride treatment resulted in the reten-
tion to the protein of 1874 dpm (31.9% of the radioactivity before 8 M urea dialysis) compared with 356 dpm (6.7% of the radioactivity) for the untreated control.

Chemical Conversion of 2-PCPA to the 2,4-DNP of Cinnamaldehyde—The product of the nitrous acid reaction had an NMR spectrum identical with that of cinnamyl chloride; no cyclopentyl proton resonances were observed. The IR spectrum, however, had additional absorances not in the spectrum of cinnamyl chloride (including a weak OH band). Furthermore, TLC analysis in three different solvent systems (20% EtOAc/n-hexane, ether, and CHCl₃) showed that the major component was cinnamyl chloride; a minor component was cinnamyl alcohol. Dichromate oxidation resulted in a product having a small doublet in the NMR spectrum at 9.67 ppm corresponding to the aldehyde proton resonance in the spectrum of cinnamaldehyde. The final product showed one spot and co-migrated with the 2,4-DNP of cinnamaldehyde by silica gel TLC in three different solvent systems: benzene:ethyl acetate (8:2) (RF₁ = 0.83); chloroform (RF₂ = 0.60); hexane:ethyl acetate (2:1) (RF₃ = 0.52). The IR spectrum was identical with that reported in the Sadler Standard Spectra (16) for the 2,4-DNP of cinnamaldehyde. The melting point, however, was 217–218 °C (literature, Ref. 12) 255 °C (decomposition) and the NMR spectrum had additional resonances which were not in the spectrum of authentic 2,4-DNP of cinnamaldehyde.

**DISCUSSION**

The product of acidic 2,4-DNPH treatment of 2-PCPA-inactivated monoamine oxidase was identified by HPLC (Fig. 1) and by TLC in two solvent systems (Fig. 2, A and B) as the 2,4-DNP of cinnamaldehyde, not of 2-phenylcyclopropane as previously reported (3, 4). Although only about 50% of the radioactivity bound to the enzyme was obtained as the 2,4-DNP of cinnamaldehyde, it was shown that the other half of the radioactivity migrated in the TLC systems with all of the radioactivity of a nonenzyme control experiment. No attempt was made to identify this nonenzyme derived product. The compound obtained after diazotization, hydrolysis, oxidation, and 2,4-DNPH treatment of 2-PCPA also was shown not to be the 2,4-DNP of 2-phenylcyclopropane as reported (3, 4), but rather had the same RF value on silica gel in three different solvent systems and had the same IR as that of the 2,4-DNP of cinnamaldehyde. Since no structure proof for their product of this chemical sequence was mentioned by Paech et al. (3, 4), it appears that the structure proposed was based solely on conjecture.

Previously, Silverman and Hoffman (17) proposed that the mechanism of monoamine oxidase-catalyzed amine oxidation involved two one-electron transfers (Scheme II). Similarly, a mechanism for inactivation of monoamine oxidase by N-(1-methyl)cylopropylbenzylamine was proposed to involve an initial one-electron transfer followed by cyclopentyl ring opening (18). Based on this mechanism and the isolation of the 2,4-DNP of cinnamaldehyde following acidic 2,4-DNPH treatment of 2-PCPA-inactivated monoamine oxidase, the proposed mechanism of inactivation of monoamine oxidase by 2-PCPA and the structure of the enzyme-inactivator adduct (2) are shown in Scheme III. It was established previously in a series of elegant experiments (3, 4) that the enzymatic group attached to 2-PCPA was not the flavin cofactor; it was suggested that an active site sulfhydryl group may instead be involved. Thiols, being both good nucleophiles and leaving groups, are known to reversibly add at neutral pH to α,β-unsaturated aldehydes (19); the product of sulfhydryl addition to cinnamaldehyde has the general structure of (Scheme III, X = S). Acid-catalyzed elimination of X to the highly conjugated cinnamaldehyde also is analogous to the rapid decomposition of β-hydroxy-β-phenylpropanaldehyde to cinnamaldehyde during the aldol condensation of acetaldehyde and benzaldehyde (20). The facility of elimination to form cinnamaldehyde, especially if the group attached were a sulfhydryl group, would explain the ease of cleavage of the enzyme-inactivator adduct under neutral denaturing conditions (3, 4). If X is the sulfur of a cysteine residue, then X could be formed by hydrogen atom abstraction of the sulfhydryl group by the flavin semiquinone radical produced in Scheme III.

Further evidence for structure 2 (Scheme III) was obtained by treating the labeled enzyme adduct solution with sodium borohydride prior to and during ethanol denaturation. After 8 M urea dialysis, the amount of radioactivity which remained bound to the enzyme was compared to the same experiment in which sodium borohydride was omitted. If elimination of HX (Scheme III) to give the resonance stabilized cinnamaldehyde is important, then prior reduction of the aldehyde should prevent elimination by increasing the pKₐ of the α-protons and decreasing the resonance stabilization of the eliminated product. Although only 31.9% of the radioactivity remained bound when sodium borohydride was included, this is 5.6 times more radioactivity (5.7%) than was found in the untreated control. These data suggest that the mechanism of inactivation of monoamine oxidase by 2-PCPA and the structure of the enzyme-inactivator adduct are consistent with that depicted in Scheme III.

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**REFERENCES**

Inactivation of Monoamine Oxidase by Tranylcypromine

Mechanism of inactivation of monoamine oxidase by trans-2-phenylcyclopropylamine and the structure of the enzyme-inactivator adduct.
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