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, dialyzed, 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-phenylcyclopropanone, but rather of cinnamaldehyde. Furthermore, denaturation of the 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 (5-8). The mechanism proposed by the Singer mechanism of inactivation of monoamine oxidase by 2-PCPA.

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, phenylcyclopropanone, 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-phenylcyclopropanone, 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-phenylcyclopropanone as reported previously (3, 4).

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials and Methods—trans-2-PCPA hydrochloride was purchased from Sigma. 2-[2-14C]PCPA, H2SO4 (specific activity 1.6 x 10⁶ dpm/μmol) was a generous gift of Dr. Carl Kaiser (Smith Kline and French Laboratories); [14C]pargyline-HCl (specific activity 3.06 x 10⁶ dpm/μmol) was kindly donated by Dr. Roy McCauley (Wayne State University). Cinnamaldehyde (Aldrich) was converted to its 2,4-DNPH 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 Tabor et al. (14).

Inactivation of Monoamine Oxidase by 2-[2-14C]PCPA—A 22 μM solution of monoamine oxidase (2.5 mg) in 1.0 ml of 50 mM HEPES, pH 7.2, containing 2-[2-14C]PCPA (final concentration of 150 μM) was incubated at 30 °C for 1 h, then was dialyzed against 3 x 500 ml of the same buffer. Radioactivity bound per amount of protein, measured by a Lowry assay, was compared to a control using [14C]pargyline as the active site titrant (15).

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 x 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 (Altex) Model 330 HPLC using an Altex ultrasphere ODS S μ C8 silica gel column and methanol:water (8:2) as eluent at a 1.0 ml/min flow rate (Tₘ = 3.6 min for cinnamic acid; Tₘ = 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 two other 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 x 20 cm) using cinnamic acid, cinnamaldehyde, 2,4-DNPH, and the 2,4-DNP of 2-PCPA.


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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-dinitrophenylhydrazone; 2,4-DNPH, 2,4-dinitrophenyldrazine; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.
cinnamaldehyde for reference. One plate was chromatographed in benzene:methanol (8:2) 

\[ R_f = 0.89 \text{ for cinnamaldehyde; } R_f = 0.73 \text{ for cinnamaldehyde; } R_f = 0.49 \text{ for } 2,4-\text{DNPH; } R_f = 0.82 \text{ for the } 2,4-\text{DNP of cinnamaldehyde.} \]

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 μM) to give a final concentration of inactivator of 150 μM. After 1 h at 30 °C, the solution was dialyzed against 2 × 500 ml of 50 μM 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 μl of buffer, to the other, 4 μl 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 to the other, 1.83 ml of cold 95% ethanol. Both were allowed to stand at 4 °C for 15 h, then each was concentrated to 450 μl with a stream of argon and diluted with 1.83 ml of 10 M urea in the above buffer (the precipitated protein mostly dissolved). The NaBH₄-treated enzyme solution contained a total of 5880 dpm inactivation to 450 μl and the control contained 6200 dpm of radioactivity. Each was dialyzed against 230 ml of 8 M urea in the above buffer at room temperature for 22 h and the entire sample (2.02 ml for the NaBH₄-treated sample, 1.93 ml for the control) was diluted with 10 ml of scintillation fluid and counted.

**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, 3.8 mmol) in 1.7 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 an orange-brown liquid (293 mg). The product was redisssolved in ether (1.5 ml) and added to a stirred solution of potassium dichromate (255 mg, 0.88 mmol) in 1.7 ml of 4.6 N H₂SO₄ added dropwise over 15 min. The mixture was stirred at room temperature for 2 h and more ether (5 ml) was added. The ether layer was separated, washed with NaHCO₃, H₂O, and brine, and dried (MgSO₄), and the solvent was evaporated to give a yellow liquid (225 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).

**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 retention of 3.5% of cinnamic acid; 0.73 for cinnamaldehyde; 0.49 for 2,4-DNPH; 0.82 for the 2,4-DNP of cinnamaldehyde. One cm strips were cut out and counted in 10 ml of scintillation fluid.

**FIG. 1.** HPLC of ethyl acetate extract after treatment of 2-[2-\[^{14}C\]PCPA inactivated monoamine oxidase with acidic 2,4-DNPH. The peak at fractions 15 and 16 corresponds to the 2,4-DNP of cinnamaldehyde. See "Experimental Procedures."

**FIG. 2.** TLC of ethyl acetate extract after treatment of 2-[2-\[^{14}C\]PCPA inactivated monoamine oxidase with acidic 2,4-DNPH. A, benzene:methanol (8:2) as eluent; B, 3% methanol in chloroform. The black spots correspond to the 2,4-DNP of cinnamaldehyde. See "Experimental Procedures."
The 2,4-DNP of cinnamaldehyde following acidic 2,4-DNPH treatment of 2-PCPA-inactivated monoamine oxidase, the mechanism of monoamine oxidase-catalyzed amine oxidation, it was suggested that an active site sulfhydryl group may have been involved. 

The compound obtained after diazotization, hydrolysis, oxidation, and 2,4-DNPH treatment of 2-PCPA was shown to be 2,4-DNP of cinnamaldehyde. Since no structure proof for 2,4-DNP of 2-phenylcyclopropanone was reported, attempts were 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-phenylcyclopropanone as reported by Silverman, R. B., and Yamasaki, R. B. (1984) in a series of elegant experiments (3, 4) that 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 semiquinon 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 pK<sub>a</sub> 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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