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Identification of the Amino Acid Bound to the Labile Adduct Formed During Inactivation of Monoamine Oxidase by 1-Phenylcyclopropylamine

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Three reactions are carried out on the reversible adduct formed when 1-phenylcyclopropylamine (1-PCPA) inactivates monoamine oxidase (MAO) in order to determine the identity of the amino acid involved in reversible adduct formation. Raney nickel treatment yields trans-β-methyl[^{14}C]styrene, the compound that would result from carbon-sulfur bond reduction of a (3-hydroxy-3-phenylpropyl)cysteine adduct. A 5,5'-dithiobis(2-nitrobenzoic acid) assay for cysteine residues indicates that upon reversible inactivation of MAO by 1-PCPA, one cysteine is lost. The third reaction involves sodium periodate and hydrogen peroxide oxidation, but no definitive result is obtained. The first two reactions provide evidence that the amino acid residue involved in reversible adduct formation is a cysteine.

It was shown previously that 1-phenylcyclopropylamine (1-PCPA)[1] is a mechanism-based inactivator (1-4) of mitochondrial MAO (5), an enzyme which contains a covalently-bound FAD cofactor and catalyzes the oxidative deamination of biogenic amines. Eight molecules of 1-[phenyl-^{14}C]PCPA are needed to irreversibly inactivate each enzyme molecule with the incorporation of one equivalent of radioactivity; an N_5-(3-oxo-3-phenylpropyl)flavin adduct (1, X = N_5-flavin) was identified as the product of the irreversible inactivation. Seven of the eight molecules of 1-[phenyl-^{14}C]PCPA were shown to form a covalent bond to an active site amino acid residue; however, this adduct decomposed with a half-life of 65 min to acrylophenone and active enzyme. The rate of irreversible inactivation was seven times slower than the rate of reversible adduct breakdown,

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[1]Abbreviations used: 1-PCPA, 1-phenylcyclopropylamine; MAO, monoamine oxidase.

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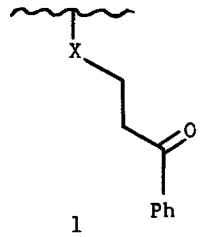
which accounts for the requirement of eight inactivator molecules per irreversible inactivation event. It was proposed that this reversible adduct had the same structure as the irreversible one, except for its point of attachment to the enzyme (1, X = amino acid residue). We report here that the amino acid



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residue (X) to which the reversible adduct is attached is a cysteine.

MATERIALS AND METHODS

Analytical Methods - Radioactivity was measured with a Beckman LS-3133T scintillation counter in 10 ml of 3a70B scintillation fluid from Research Products International. Optical absorbance was measured with a Perkin-Elmer Lambda 1 UV/VIS spectrophotometer. Thin-layer chromatography was performed on silica gel 60 coated plastic plates (Merck) using 3:1 n-hexane:ethyl acetate as the solvent system. High performance liquid chromatography was carried out on a Beckman Model 330 liquid chromatograph with an Alltech analytical 5μ silica gel column, eluting with 3:1 n-hexane-ethyl acetate at 2 ml/min. The instrument was equipped with a UV detector (254 nm) and a Hewlett-Packard 3390A recording integrator. Protein concentration determinations were carried out using the Pierce BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, Illinois). The 5,5'-dithiobis(2-nitrobenzoic acid) assay of Fernandez Diez et al. (6) was used to determine the number of enzyme sulfhydryl groups. The method of Cavallini et al. (7) was used for sodium borohydride treatment of the enzyme prior to 5,5'-dithiobis(2-nitrobenzoic acid) assay.

Reagents - n-Propylbenzene, trans-β-methylstyrene, 1-phenylpropanol, 5,5'-dithiobis(2-nitrobenzoic acid), Raney nickel (aqueous slurry), and sodium periodate were bought from Aldrich. The hydrochloride salts of benzylamine, 1-PCPA, and 1-[phenyl-^{14}C]PCPA were prepared as previously reported (5).

Enzymes and Assays - Mitochondrial monoamine oxidase (EC 1.4.3.4) was isolated and assayed as previously described (5). Pronase (protease, type XIV, a non-specific protease from S. griseus) was obtained from Sigma.

Release of Radioactivity from MAO Reversibly Inhibited by 1-[phenyl-^{14}C]PCPA upon Treatment with Raney Nickel - 1-[phenyl-^{14}C]PCPA (10 mM) in 50 mM potassium phosphate, pH 7.2 buffer (125 μl) was added to 150 μM MAO in the same buffer (125 μl) and was incubated at 25°C for 90 min. Sodium borohydride (2 mg) in 0.2 N sodium borate, pH 9.5 buffer (250 μl) was added, and the solution was incubated in the dark for 2.5 h at room temperature. The reduced enzyme solution was dialyzed against 20 mM potassium phosphate, pH 7.2 buffer (4 x 80 ml, 1.5 h each change), then the protein was denatured by the addition of trichloroacetic acid (50 mg). The denatured enzyme was pelleted by centrifugation in a Beckman Microfuge B for 30 sec, redispersed in 5% trichloroacetic acid (750 μl), and pelleted in the same manner. The pellet was then washed successively with methanol (750 μl), chloroform (2 x 750 μl), methanol (750 μl), and 50 mM potassium phosphate, pH 7.9 buffer (2 x 750 μl). The washed protein was dispersed in 850 μL of 50 mM potassium phosphate, pH 7.9 buffer, and digested with Pronase (1.5 mg) at 37°C for 16 h. Additional Pronase (1.5 mg) was added,

and the digestion was continued for another 24 h. After centrifugation to remove a small white pellet, a portion (600 µl) was removed and an aqueous slurry of Raney nickel (600 µl) was added. The sample was heated at 50°C for 6 h with stirring; then after cooling, the sample was extracted with ether (7 x 1 ml). Portions of the extracts and the residue were removed and the radioactivity was determined. The ether was evaporated and the extracted material was analyzed by thin layer chromatography and high performance liquid chromatography using n-propylbenzene, trans-β-methylstyrene, and 1-phenylpropanol as standards. The R_f values are 0.66, 0.62, and 0.40, and the retention times are 1.75, 1.95 and 3.22 min., respectively. A control also was carried out substituting 200 mM sodium borate, pH 10 buffer for the Raney nickel suspension. 1-Phenylpropanol was treated with Raney nickel or pH 10 buffer, extracted, and chromatographed in the same manner as described above.

Effect of Oxidizing Reagents on Release of Radioactivity from MAO Reversibly Inhibited by 1-[phenyl-^{14}C]PCPA - MAO was inactivated as described above, then was applied onto Sephadex G-25 (0.7 x 12 cm), eluting with water at a flow rate of 0.5 ml/min, and collecting a 550 µl fraction containing the enzyme. A portion (250 µl) was removed and added to a buffered solution of sodium periodate (250 µl, 0.2 M final concentration, pH 7.2) and urea (8 M final concentration). Aliquots (50 µl) were removed at 0, 0.25, 0.5, 1, 1.5, 2, 2.75, 3.5, and 24 h and applied onto Sephadex G-25 (0.5 x 6.5 cm), eluting with water at a flow rate of 0.5 ml/min. Five fractions were collected, and both the radioactivity released from the enzyme and the radioactivity remaining on the enzyme were determined. A control was carried out in the same manner as described above, without the sodium periodate. The same procedure was carried out substituting 20% H_2O_2 in 1 M potassium phosphate buffer, pH 7.2 for the sodium periodate solution.

RESULTS AND DISCUSSION

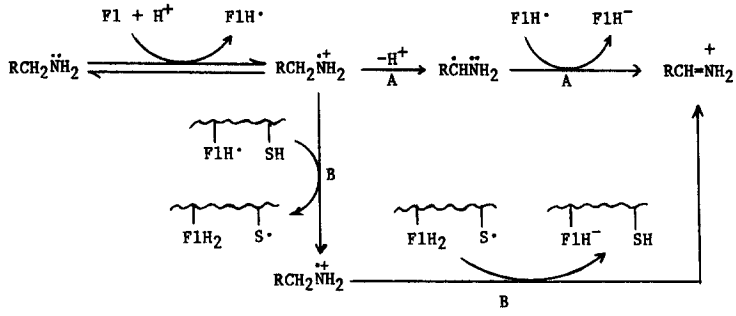
Three different approaches were taken to identify the amino acid to which 1-PCPA reversibly binds. It was shown previously (5) that sodium borohydride reduction of the reversible adduct formed from 1-[phenyl-^{14}C]PCPA with MAO stabilized the adduct and prevented its spontaneous release; this is what would be expected if the ketone in 1 were reduced to an alcohol. Raney nickel is known to cleave carbon-sulfur bonds specifically (8) and Danenberg and Heidelberger (9) found that this reagent only reduced cysteine and methionine residues in proteins. When the sodium borohydride-reduced and Pronase digested reversible adduct formed from 1-[phenyl-^{14}C]PCPA and MAO was treated with Raney nickel, one radioactive compound was formed, which was identified by thin-layer and high performance liquid chromatography as trans-β-methylstyrene. Cleavage of the carbon-sulfur bond of the reduced adduct was predicted to give 1-phenyl-1-propanol, but it was shown in a non-enzymatic control reaction that under the same conditions used for the Raney nickel reduction of the labeled enzyme, 1-phenyl-1-propanol was dehydrated to trans-β-methylstyrene. This result supports attachment to a cysteine residue. trans-β-Methylstyrene was the

only radioactive compound that was extracted from the Raney nickel reaction, but it accounted for only 30% (11,580 cpm) of the radioactivity bound to the peptide fragment (38,300 cpm). Danenberg and Heidelberger (9) inactivated thymidylate synthetase with labeled 5-fluoro-2'-deoxyuridylate and found that Raney nickel treatment released only 10% of the bound radioactivity. However, extraction of the Raney nickel gave an additional 40% of the same radioactive compound released originally. Their conclusion, as is ours, is that the remainder of the radioactivity is adsorbed onto the catalyst. In the absence of Raney nickel, only 3% (1190 cpm) of the radioactivity was organic extractable, which did not migrate on silica gel as trans-β-methylstyrene.

The second experiment carried out was a determination of the effect of reversible inactivation on the total number of cysteine residues in MAO. A 5,5'-dithiobis(2-nitrobenzoic acid) assay of denatured native enzyme revealed the presence of 6.2 ± 0.1 cysteine residues (4 experiments). Upon treatment with 1-PCPA, only 5.2 cysteine residues could be detected under the same conditions (2 experiments).[2] The same results were obtained when the native and inactivated enzymes were pretreated with sodium borohydride prior to the sulfhydryl assay (2 experiments).

The third experiment carried out was based on the hypothesis (5) that the reversibility of this adduct is the result of the leaving group ability of the amino acid to which it is attached. Thiolate is a good leaving group, but sulfinate is even better (10). Since sodium periodate and hydrogen peroxide are mild oxidzing agents known to convert sulfides to sulfoxides (11,12), oxidation of alkylated cysteine should increase the leaving group ability of the bound amino acid. However, there was no significant difference in the rate of release of radioactivity from 1-[phenyl-^{14}C]PCPA-inactivated MAO, with or without sodium periodate or hydrogen peroxide treatment. The conversion of sulfides to

[2]With an older preparation of the enzyme, only 3.7 ± 0.4 cysteine residues (5 experiments) were detected, but in those cases, 1-PCPA inactivation gave a protein containing only 2.6 ± 0.2 cysteines (3 experiments). The lower cysteine content in this batch of enzyme may be the result of partial cysteine oxidation; however, this enzyme was consumed before the effect of sodium borohydride treatment on the number of free cysteines could be determined.



Scheme 1. Mechanisms for MAO-Catalyzed Amine Oxidation. Fl is oxidized flavin.

sulfoxides occurs at room temperature in 20 min to an hour (12), and the experiment to determine the rate of radioactivity release was carried out over 3.5 hours. Therefore, since there is no change in the rate of radioactivity release, it suggests that the rate determining step may be removal of the α-proton, rather than elimination of the thiolate (sulfinate). This result, then, is ambiguous as to which amino acid is involved. When attachment of 1-PCPA occurs at the N_5-position of flavin to give the irreversible adduct, elimination of the flavin anion is a much slower process (5).

The results of the first two of these tests are consistent with cysteine being the amino acid residue to which 1-PCPA reversibly binds. This result further supports the hypothesis (5) that the flavin radical anion generated from one-electron transfer of a substrate or inactivator amine to FAD could be in equilibrium with an amino acid radical. Since thiols are excellent hydrogen atom donors (13), a cysteine residue is a reasonable candidate for this exchange process. It is not clear whether this hypothetical hydrogen atom exchange reaction between FAD radical anion and a cysteine residue is involved in enzyme catalysis of normal substrates or only in reactions of poor substrates such as 1-PCPA. If it is involved, this work may support a hydrogen atom abstraction mechanism (Scheme 1, pathway B) rather than the proton transfer-electron transfer mechanism (Scheme 1, pathway A) more commonly written for amine oxidation mechanisms. Possibly, either the flavin semiquinone or the thiyl radical is capable of being involved in catalysis, and this may account for the broad specificity of MAO.

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