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Inactivation of Monoamine Oxidase B by 1-Phenylcyclopropylamine: Mass Spectral Evidence for the Flavin Adduct

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Abstract—Incubation of 1-phenylcyclopropylamine with bovine liver MAO (MAO B), followed by complete enzymatic digestion to single amino acid residues and subsequent analysis by on-line liquid chromatography–electrospray ionization mass spectrometry, was used to investigate the resulting flavin adduct structure.

Monoamine oxidase (MAO; EC 1.4.3.4) contains a covalently-bound flavin and is responsible for the catalytic oxidative deamination of biogenic and xenobiotic amines (Scheme 1).[1,2] MAO is known to act on important neurotransmitters, such as serotonin and norepinephrine, as well as to oxidize benzylamine, phenylethylamine, and the tertiary amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) at significant rates.[3–5] Therefore, the inhibition of this enzyme has been widely studied for application as a clinical treatment of neurological disorders such as Parkinson's disease and depression.[6–9]

Although monoamine oxidase has been studied for more than half a century, certain aspects of the enzyme, such as its substrate binding site and chemical mechanism, are still the focus of debate. Of the mechanisms proposed for MAO B, including hydride transfer,[10] carbanion,[11] group transfer,[12] hydrogen atom transfer (HAT),[13] and one-electron (radical),[14] chemical evidence is strongest for the radical mechanism.[15]

Mechanism-based inactivators of MAO have been studied both as potential drug targets and for the information they provide regarding the mechanism of the enzyme. A variety of cyclopropyl amines have been shown to act as mechanism-based inactivators of MAO B.[16–22] It is proposed that, following single electron transfer from the amine to the flavin, the cyclopropyl ring undergoes a ring cleavage reaction, leading to covalent attachment to an active site cysteine residue or to the flavin cofactor.[23,24] In the case of the inactivator 1-phenylcyclopropylamine (1-PCPA), Zieske and Silverman suggested the formation of both a cysteine adduct (reversible) and a flavin adduct (irreversible),[19,20] although there was no direct evidence for the covalent flavin adduct formation.

We report here the cleavage of the 1-PCPA-modified flavin from MAO B; isolation and subsequent assignment of its structure, using liquid chromatography–electrospray ionization mass spectrometry (LC/ESI-MS) support the formation of a covalent 1-PCPA-flavin adduct.

MAO B (47 μM; specific activity 5.80 units/mg; isolated from beef liver as previously reported[25]) was inactivated with 10 mM 1-PCPA by the procedure reported previously by Silverman and Zieske.[19] After 9 days of incubation at room temperature, the MAO B enzyme control and inactivated samples were each subjected to a PD-10 column (Pharmacia Biotech, Sephadex 25 M), and were eluted with 50 mM sodium phosphate buffer,

Scheme 1. General reaction for the oxidation of amines catalyzed by MAO.

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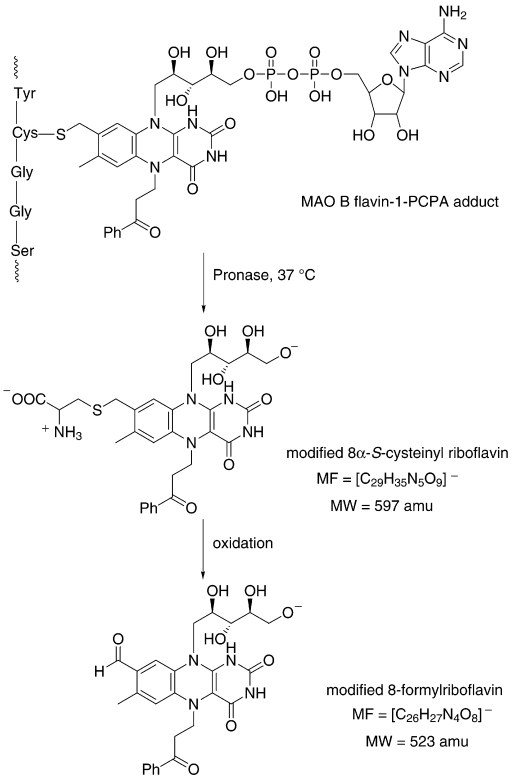
pH 7.2. After concentration to 750 μL, Pronase (Boehringer-Mannheim) (0.77 mg, 50 μL) was added to each of the MAO B control and inactivated samples. After incubation in the dark at 37 °C for 18 h, additional Pronase was added (0.77 mg, 50 μL). Following an additional 25 h incubation, the samples were placed in liquid N_2 to stop the digestion, microfuged [14,000 rpm (16,000*g*), 15 min], then the yellowish supernatant was removed and concentrated. Aliquots of 15 μL each were injected onto a Hypersil BDS C18 column (2×250 mm) connected to a Micromass Quattro II mass spectrometer (Fisons Instruments, Manchester, UK). The adduct was eluted with the following system: mobile phase A 100% water; mobile phase B 100% methanol. Gradient: initial, 6% B; 14.50 min, 20% B; 30 min, 20% B; 30.5 min, 90% B; 35.5 min, 90% B; 36 min, 6% B. The flow rate was 0.190 mL/min with a stop time of 50 min. A solvent delay of 5 min was used to prevent salt contamination of the mass detector. Negative ion mode electrospray mass spectra were acquired. Triethylamine (1 mM) in methanol was added post column to assist in the sample ionization. Instrument tuning parameters (ESI^-) were used along with the following operational parameters: capillary 3.08 kV; cone voltage 40 V; and source temperature 140 °C. The mass range was set at 500–1500 amu, with a scan rate of 2 s/scan.

Pronase (EC 3.4.24.31), a mixture of at least 10 proteases isolated from *Streptomyces griseus*, was used for the MAO B digestion. Pronase is nonspecific in its action of complete protein degradation, breaking down both denatured and native proteins into individual amino acids.[26–28] Because Pronase is stable and active in the same buffer as the MAO B inactivation incubation, it could be added directly to the incubation mixture without the extensive sample preparation required for trypsin and chymotrypsin digestion.

The MAO B flavin cofactor exists as FAD; however, under digestion conditions, the phosphodiester bond cleaves (hydrolyzes) to yield riboflavin. 8α-*S*-Cysteinylriboflavin was synthesized by Ghisla and Hemmerich and was shown to have the same properties as those of the product isolated from the digestion of MAO.[29] In many cases, 8α-*S*-cysteinyl thioethers have been shown to undergo autooxidation and cleavage to generate 8-formylflavin as a product (Scheme 2).[30–33]

Two peaks of interest appeared in the HPLC chromatogram of the inactivated sample that were not present in the control. A peak with the retention time of 9.7 min, *m*/*z* 597.3, was found, which corresponds to a



Scheme 2. Formation of the flavin adducts observed by mass spectrometry after treatment of 1-PCPA-inactivated MAO B with Pronase.

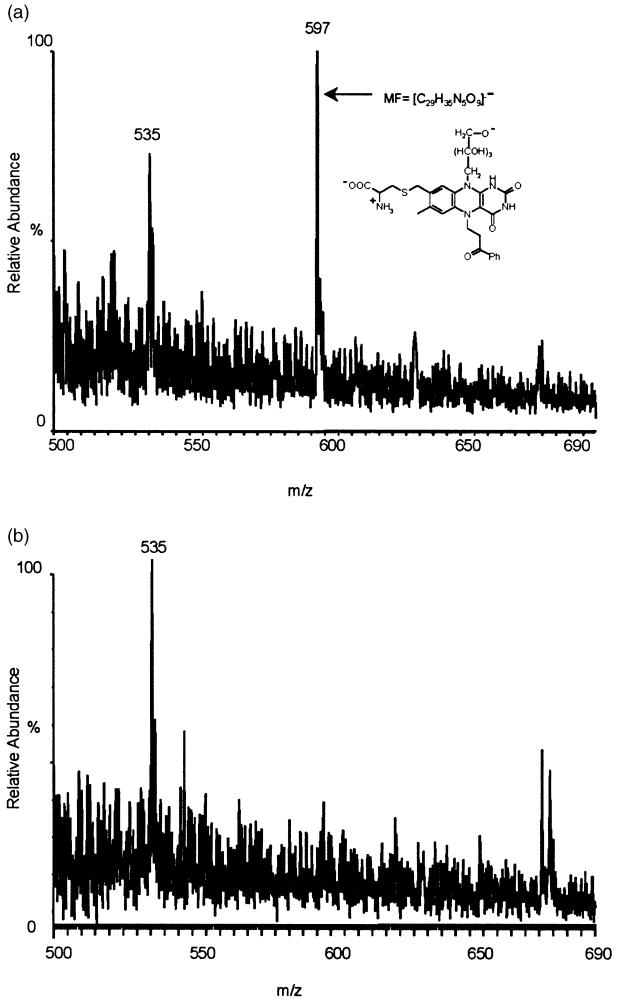


Figure 1. (a) Negative mode electrospray ionization mass spectrum of MAO B inactivated with 1-PCPA after digestion with Pronase [8α-*S*-cysteinyl riboflavin + 1-PCPA]; (b) negative mode electrospray ionization mass spectrum of MAO B digest (control, no inactivator), after digestion with Pronase.

cysteine bound to 1-PCPA-modified riboflavin (Fig. 1a). Upon Pronase digestion, the peptide bonds surrounding the FAD cofactor and the phosphodiester bond of the FAD were hydrolyzed, as reported by Ghisla and Hemmerich for the native enzyme.[29] This peak was not present in the control (Fig. 1b). A second peak, m/z 523.9, was found in the inactivated sample, which corresponds to 1-PCPA-modified 8-formylriboflavin (Fig. 2). Mild oxidation of the 8-cysteinyl riboflavin thioether[30–33] would yield modified 8-formylriboflavin (523 amu). Scheme 2 shows these transformations.

The generation of these adducts can be rationalized based on previous mechanistic studies of MAO B with 1-PCPA. Radiolabeling experiments by Silverman and Zieske suggested the formation of an irreversible flavin adduct when MAO B was inactivated with 1-[phenyl-^{14}C]PCPA.[19] The one-electron mechanism shown in Scheme 3 was proposed, although there was no direct evidence for the formation of the covalent 1-PCPA-flavin adduct. The electrospray ionization mass spectrometry experiments described here provide direct evidence for the formation of this covalent flavin adduct.

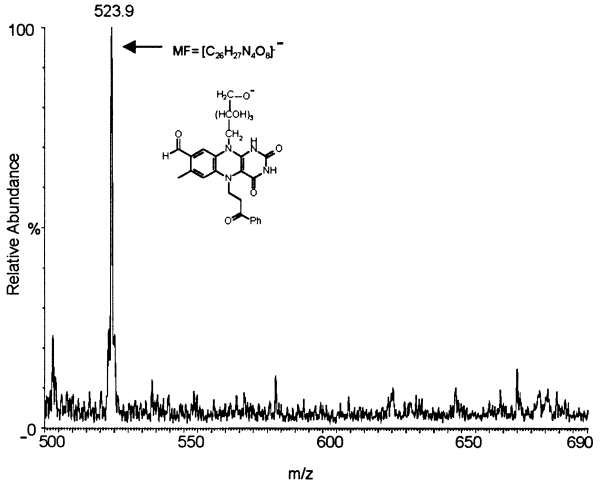
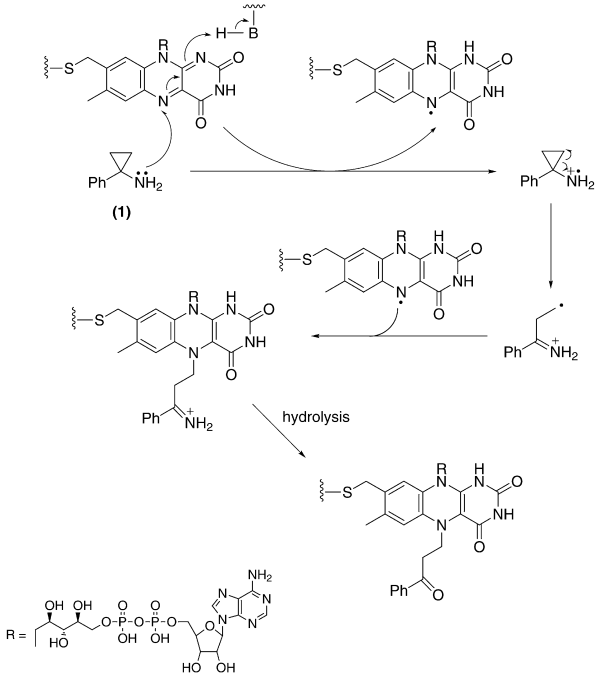


Figure 2. Negative mode electrospray ionization mass spectrum of MAO B inactivated with 1-PCPA, after digestion with Pronase [8-formylriboflavin + 1-PCPA].



Scheme 3. One-electron mechanism proposed for the flavin adduct formed upon inactivation of MAO B with 1-PCPA.

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