Identification of the Active Site Cysteine in Bovine Liver Monoamine Oxidase B

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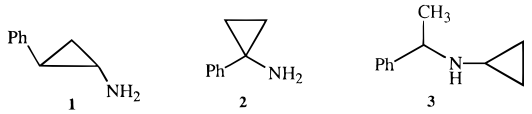
Monoamine oxidase (MAO, EC 1.4.3.4) is a flavoenzyme that is located in the outer mitochondrial membrane and catalyzes the oxidation of various amine neurotransmitters and dietary amines to the corresponding imines, which are nonenzymatically hydrolyzed to aldehydes.[1] Although this enzyme has been recognized for almost 70 years,[2] there are no reports of its three-dimensional structure. MAO exists in two isozymic forms, termed MAO A and MAO B, which have 70% sequence identity as deduced from their cDNA clones.[3] The active forms of the enzymes are homodimers with subunit molecular weights, determined from their cDNA structure, of 59 700 and 58 800, respectively. The genes for both MAO A and MAO B have strikingly similar structures; both consist of 15 exons and exhibit identical exon−intron organizations, suggesting that MAO A and MAO B are derived from duplication of a common ancestral gene.[4] Selective inhibitors of MAO A are used to treat depression,[5] whereas MAO B inhibitors are used in the treatment of Parkinson's disease.[6]

The FAD cofactor is covalently bound at the active site of both isozymes via a C-8α linkage to a cysteine residue in the pentapeptide Ser-Gly-Gly-Cys(FAD)-Tyr, which has been isolated from both MAO A and MAO B after digestion with chymotrypsin and trypsin.[7] Nine cysteines were found in the deduced amino acid sequences of both human liver MAO A and MAO B.[3] Each cysteine residue of MAO A and MAO B was mutated to a serine residue, and it was found that, in addition to the FAD binding site (Cys-406 in MAO A and Cys-397 in MAO B), Cys-374 plays an important role in the catalytic activity of MAO A, whereas Cys-156 and Cys-365 are important for MAO B activity.[8] However, it could not be determined from these studies whether these cysteine residues were in the active site or were important for the appropriate conformation of the enzymes.

trans-2-Phenylcyclopropylamine (**1**; tranylcypromine) is a mechanism-based inactivator[9] of MAO that was introduced clinically for the treatment of depression in 1961.[10] A series of experiments demonstrated that **1** does not become attached to the active site flavin of MAO B, and it was conjectured that it may be attached to a cysteine residue.[11−13] The inactivation



of MAO B by 1-phenylcyclopropylamine (**2**)[14] was shown to produce a covalent adduct to an active site cysteine residue.[15] *N*-Cyclopropyl-α-methylbenzylamine (**3**) was demonstrated to inactivate MAO B without attachment to the flavin; attachment was presumed to be at an active site *amino acid residue*.[16] Compounds **1**−**3** were shown to be active site directed inactivators by virtue of the fact that the substrate (benzylamine) protects the enzyme from inactivation. If, in fact, these inactivators become attached to a cysteine residue and this residue can be identified, then it would be apparent which cysteine was in the active site. In this paper, bovine liver MAO B is inactivated with **3** and the residue to which it becomes attached is identified. This provides the first direct identification of an active site residue for this enzyme other than those attached to the flavin cofactor.

Beef liver mitochondrial MAO B was purified by the known method.[17] After the enzyme was inactivated with **3** and sodium borohydride reduced to stabilize the adduct,[18] it was sent to the Harvard Microchemistry Laboratory (William S. Lane, Director), along with a sample of the enzyme that had not been inactivated but was reduced, for digestion with Lys-C endopeptidase, HPLC separation of the peptides, and sequence analysis.[19]

Peptide maps from Lys-C digests of uninactivated and inactivated MAO B are shown in Figures 1 and 2, respectively. At times up to 70 min, all of the peaks in the HPLC trace of inactivated enzyme coincide with peaks in the chromatogram of the uninactivated sample, except for the peaks indicated with arrows. These peaks were isolated and sequenced. The A peptide was identified as the octapeptide Lys-Leu-X-Asp-Leu-

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(18) Into 150 μL of 130 μM bovine liver MAO B in 50 mM sodium phosphate buffer (pH 7.2) were added 150 μL of 50 mM Tris buffer (pH 9.0) and 150 μL of 20 mM **3** in distilled water. The resulting mixture was incubated at room temperature for 3 h, and then 450 μL of 0.2 N sodium borate buffer (pH 9.2) was added with 30 μL of sodium borohydride in 0.1 N sodium hydroxide (1 mg/10 μL). After standing in the dark overnight, the solution was dialyzed against 8 M urea in 20 mM sodium phosphate buffer (pH 7.2, 3 × 400 mL, 2 h each) and then against 8 M urea in 100 mM ammonia bicarbonate solution (pH 8.3, 2 × 400 mL, 2 h each).
(19) The samples were dried in a Savant SpeedVac then were redissolved in 50 μL of 200 mM ammonium bicarbonate buffer, pH 8.0. A 5 μL aliquot of 45 mM DTT (Calbiochem) was added to this solution, which was heated to 50 °C for 15 min. After the solution was cooled to room temperature, a 5 μL aliquot of iodoacetamide (Sigma) was added. After the solution was allowed to stand for 15 min, it was diluted to a concentration of 100 mM ammonium bicarbonate by the addition of 45 μL of distilled water, and then it was subjected to enzymatic digestion. An appropriate amount of Wako Lys-C (sequencing grade) in <5 μL was added to the reduced and alkylated samples to maintain an enzyme:substrate ratio equal to 1:25 (wt/wt), and the digestion was allowed to proceed for 24 h at 37 °C. The resulting peptides were separated by narrow-bore HPLC (C18) using the following elution program at 0.345 mL/min: 0−63 min, 95% solvent A, 5% solvent B; 63−95 min, 67% solvent A, 33% solvent B; 95−105 min, 40% solvent A, 60% solvent B; 105−107 min, 20% solvent A, 80% solvent B (flow rate changed at 106 min to 0.433 mL/min); 107−113 min, 5% solvent A, 95% solvent B. Solvent A is 0.060% TFA in water; solvent B is 0.055% TFA in acetonitrile.

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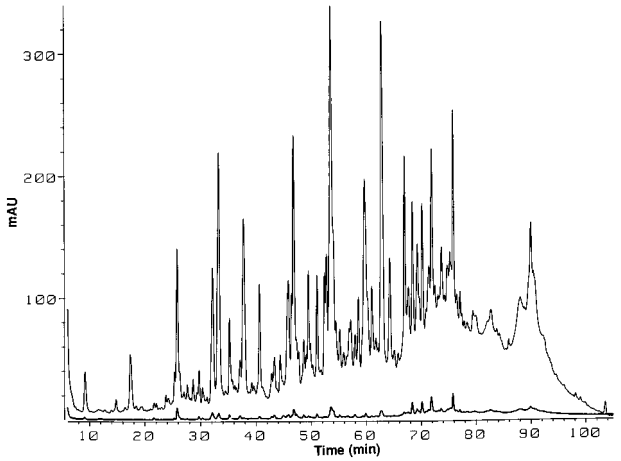


Figure 1. HPLC of Lys-C digest of monoamine oxidase B under the same conditions as the inactivated enzyme. The lower chromatogram was detected at 277 nm, and the upper chromatogram was detected at 205 nm.

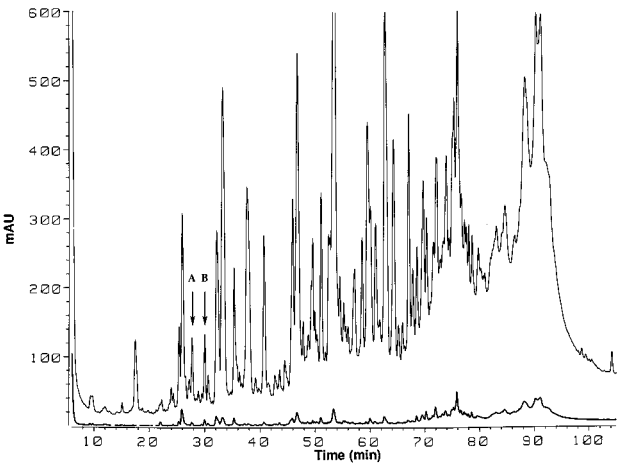


Figure 2. HPLC of Lys-C digest of monoamine oxidase B inactivated with **3** and sodium borohydride reduced. The lower chromatogram was detected at 277 nm, and the upper chromatogram was detected at 205 nm.

Tyr-Ala-Lys, where the X represents a modified amino acid. On the basis of the partial amino acid sequence of bovine liver MAO B,[20] the modified amino acid is Cys-365. This octapeptide was shown[20] to be very similar to the corresponding octapeptides in the deduced sequences from cloned human MAO A (Lys-Ile-Cys-Glu-Leu-Tyr-Ala-Lys) and cloned human MAO B (Lys-Leu-Cys-Glu-Leu-Tyr-Ala-Lys). The corresponding conserved cysteine residues in the cloned enzymes are Cys-374 and Cys-365 for MAO A and B, respectively. There was not enough of peptide B to make an adequate identification, but it appeared to contain peptide A.

Further support for the structure of the modified cysteine residue was obtained at the Harvard Microchemistry Laboratory with matrix-assisted laser desorption ionization (MALDI) mass

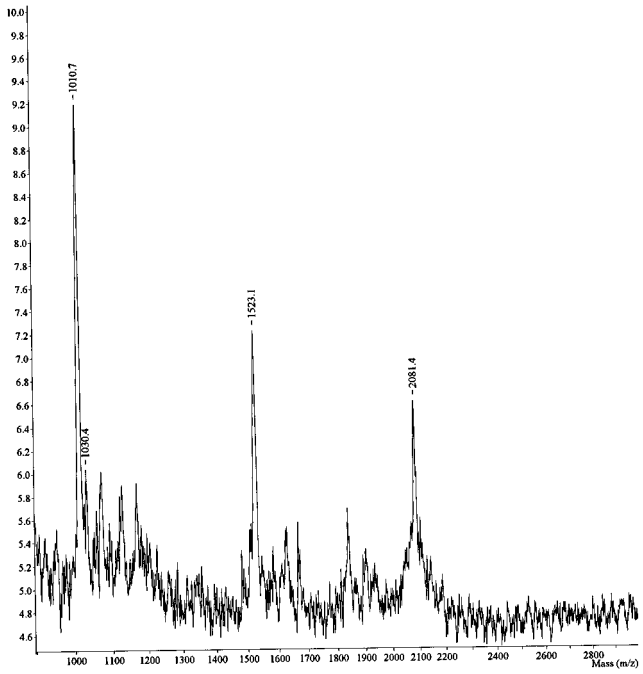
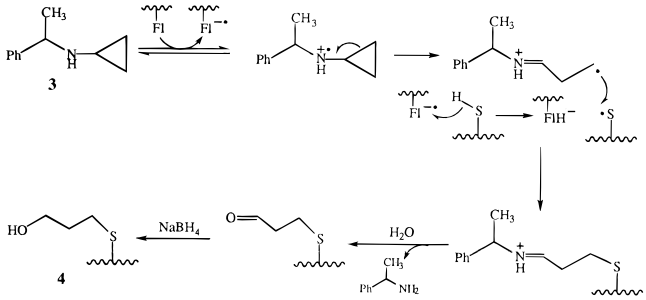


Figure 3. MALDI-TOF mass spectrum of peptide A from Figure 2.

Scheme 1



spectrometry (Figure 3).[21] The major peak from the isolated peptide was found to have a mass of 1010.7 amu. According to the mechanism proposed for inactivation of MAO B by **3**[16] (Scheme 1 is a modification of the previously-proposed mechanism[16]), following sodium borohydride reduction to stabilize the adduct, the mass of the modified octapeptide (**4**) should be 1010.55 amu (952.505 for the octapeptide minus 1.008 for the proton from the modified cysteine thiol group plus 59.051 for $CH_2CH_2CH_2OH$ from the inactivator). This establishes the structure of the modified octapeptide as well as provides further support for the proposed inactivation mechanism.

In summary, *N*-cyclopropyl-*N*-α-methylbenzylamine (**3**), a mechanism-based inactivator of bovine liver MAO B, labels Cys-365, which corresponds to Cys-374 and Cys-365 in human MAO A and MAO B, respectively, and identifies these cysteine residues to be in the active site of the respective isozymes.

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