Monoamine Oxidase B Catalysis in Low Aqueous Medium. Direct Evidence for an Imine Product

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Monoamine oxidase (MAO, EC 1.4.3.4) catalyzes the oxidative degradation of a wide variety of amine substrates to their corresponding aldehydes. Inhibitors of MAO have been used clinically as antidepressants[1] and in the treatment of Parkinson's disease.[2,3] It has long been assumed that the actual product released from the active site of the enzyme was the aldimine and that hydrolysis of the aldimine occurred in a nonenzymatic step. According to an abstract in *Federation Proceedings*,[4] when MAO and *N*-methylbenzylamine were incubated and treated with sodium borotritide, tritiated substrate could be detected, suggesting the formation of benzylidenemethylamine. Contrary to this finding, however, are studies on benzylamine oxidation by MAO,[5] which indicate that ammonium ion is released after O_2 binding, implying that hydrolysis occurs on the reduced enzyme–product complex. More recently, stopped-flow kinetic studies[6] have shown that the aldimine products in the oxidation of *p*-(*N*,*N*-dimethylamino)benzylamine and *N*-methyl-*p*-(*N*,*N*-dimethylamino)benzylamine are released and hydrolyzed nonenzymatically. In this communication, we present direct chemical evidence that the imine is released as the product of MAO-catalyzed amine oxidation, and we demonstrate that MAO is catalytically active in a low aqueous medium.

Over approximately the last 10 years, particularly as a result of the work of Klibanov and co-workers, it has been found that enzymes can function in anhydrous and low water media.[7-11] If an imine is the product of amine oxidation catalyzed by MAO, then the use of MAO in an organic solvent would permit direct isolation and identification of the imine prior to its hydrolysis to the corresponding aldehyde. Recently, MAO B was found to be catalytically active in a variety of organic solvents.[12] Oxidation of *N*-methylbenzylamine in benzene containing 1% (v/v) water (so that water was available for the enzyme if needed in the reaction; this is a hetereogeneous solvent mixture) produced *N*-benzylidenemethylamine as the sole product; no benzaldehyde was detected. The imine product formation was monitored by capillary gas chromatography.[13] *N*-Methylbenzylamine is as good a substrate for MAO B in buffer as is benzylamine.[14] The K_m and k_{cat} values for *N*-methylbenzylamine in 50 mM sodium phosphate buffer (pH 7.2) were 0.152 mM and 117 min^{-1}, respectively, while in benzene values of 2.3 mM and 124 min^{-1} were obtained. This observation, that the K_m is altered but that the k_{cat} remains virtually unchanged, correlates well with a competition of the hydrophobic aromatic ring of *N*-methylbenzylamine for the bulk solvent and for the hydrophobic pocket of the active site of the enzyme.

Inspection of the velocity curves (a representative velocity curve is shown in Figure 1) for the reaction between *N*-methylbenzylamine and MAO B reveals that significant product inhibition occurs as the concentration of the product increases. It has been reported that *p*-hydroxybenzaldehyde is an uncompetitive inhibitor of MAO B and that it acts as a product analogue,[15] suggesting that the aldimine is the turnover product,

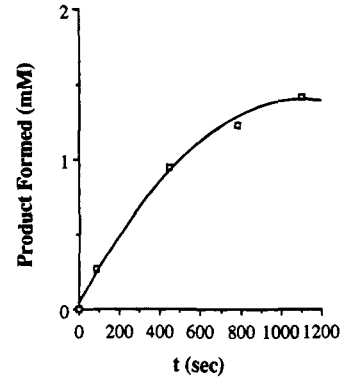

Figure 1. Oxidation of 7.8 mM *N*-methylbenzylamine by MAO (1.8 μM) in benzene containing 1% (v/v) water. The temperature was held constant at 25 °C.

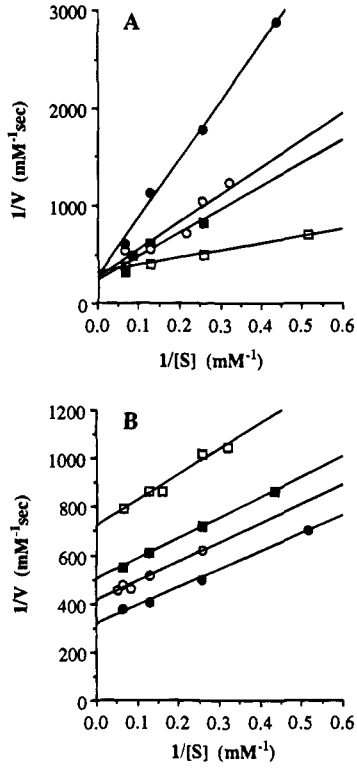

Figure 2. (A) Inhibition of MAO (1.8 μM) by 0 (□), 2.4 (■), 3.9 (○), and 8.0 (●) mM benzylidenemethylamine in benzene containing 1% (v/v) water. (B) Inhibition of MAO (1.8 μM) by 0 (●), 4.9 (○), 14.7 (■), and 19.6 (□) mM benzaldehyde in benzene containing 1% (v/v) water. The temperature was maintained at 25 °C for all experiments.

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(13) Stock enzyme (10 μL, 15–25 mg/mL in 50 mM sodium phosphate buffer, pH 7.2) was pipetted into a vial containing varying concentrations of inhibitor and substrate (see legends for Figures 1 and 2) in 990 μL of benzene, held at 25 °C with a Lauda K-2/RD constant temperature bath. The enzyme–inhibitor–substrate mixture was sonicated in an ultrasonic cleaning bath, then 1 mL aliquots were removed periodically and analyzed by capillary gas chromatography (Hewlett Packard 5730A gas chromatograph equipped with an Alltech 30 meter, 32 μm i.d. SE-30 capillary column).
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not the aldehyde. To date no studies have been able to directly address the question of product inhibition by the aldimine product because of its high instability in aqueous buffer.[16] However, in low aqueous organic solvent media, the imine is stable;[17] consequently, benzylidenemethylamine was tested as an inhibitor of MAO B in benzene containing 1% (v/v) water. Double reciprocal plots revealed that benzylidenemethylamine is a competitive inhibitor with respect to *N*-methylbenzylamine (Figure 2A), suggesting that the product binds at the active site of the enzyme. The K_i value was 2 mM, similar to the K_m obtained for the substrate in the same solvent. However, when the same analysis was carried out in the presence of benzaldehyde instead of benzylidenemethylamine, uncompetitive inhibition was observed (K_i = 15 mM) (Figure 2B).

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(16) The $t_{1/2}$ for hydrolysis of benzylidenemethylamine in 50 mM sodium phosphate (pH 7.2) buffer was measured to be less than 18 s.

(17) No hydrolysis of the imine control was detected by gas chromatography during the 6 h experiments.

The data presented in this communication show that the aldimine is the product of MAO oxidation of amines and that hydrolysis to the corresponding aldehyde, therefore, does not occur on the reduced enzyme. To the best of our knowledge, this is the first example of the use of a nonaqueous system for such an analysis and should be applicable to test other amine oxidases or other enzyme systems with hydrolytically labile products.

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Supplementary Material Available: Secondary plots in the determination of K_i values (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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