Inactivation of Monoamine Oxidase by (Aminomethyl)cubane. First Evidence for an α-Amino Radical During Enzyme Catalysis

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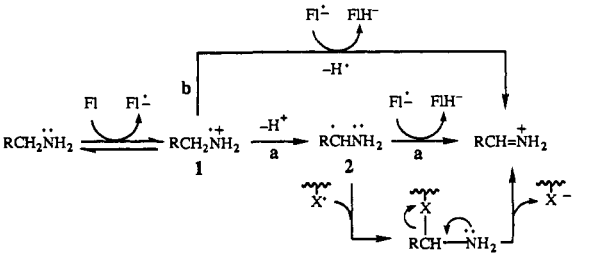
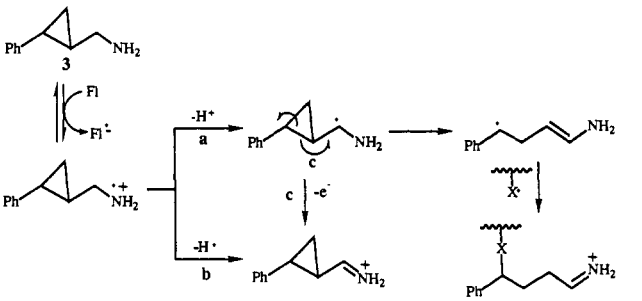
Received June 21, 1993

Monoamine oxidase (EC 1.4.3.4; MAO), a flavin-dependent enzyme that catalyzes the oxidation of a variety of amine neurotransmitters, exists in two isozymic forms known as MAO-A and MAO-B.[1] Evidence from studies with cyclopropylamines,[2] cyclobutylamines,[3] and silicon-containing substrate analogues[4] suggests that oxidation of the amine to an amine radical or radical cation (**1**) is a reasonable early step in catalysis (Scheme I). Recently, we attempted to determine if proton transfer (pathway a), giving the α-amino radical **2**, followed by second electron transfer or direct hydrogen atom transfer (pathway b), was responsible for formation of the product iminium ion.[5] Because of the exceedingly rapid rate of cyclopropane ring opening in solution for the *trans*-2-phenylcyclopropane carbinyl radical,[6] *trans*-2-phenyl(aminomethyl)cyclopropane (**3**, Scheme II) was designed to probe for the intermediacy of the corresponding α-amino radical during enzyme catalysis.[5] If that radical were formed, then it was predicted that cyclopropane ring cleavage would occur, resulting in a radical that should inactivate the enzyme by attachment to an active site radical (Scheme II, pathway a). No inactivation occurred, and the only metabolite detected was *trans*-2-phenylcyclopropanecarboxaldehyde, suggesting that the major pathway in Scheme II is pathway b. The other explanation, however, for not observing cyclopropane ring cleavage is that pathway a is the predominant pathway, but second electron transfer to the iminium ion (pathway c) is faster than ring cleavage. Three rationalizations can be offered for why this could occur: (1) the amino group stabilizes the α-amino radical sufficiently so that second electron transfer becomes faster than ring opening; (2) the orbitals of the cyclopropane bond that must break do not overlap sufficiently with the adjacent orbital containing the radical; and (3) second electron transfer is exceedingly rapid. Recently, it was shown that no ring opening occurred during incubation of cytochrome P-450[7] or methane monooxygenase[8] with *trans*-2-phenylcyclopropane analogues; both of these enzymes are believed to involve radical mechanisms as well. It is known that adjacent stabilizing groups can slow down cyclopropane ring cleavage,[9] but no direct measurement for the stabilizing effect of an α-amino group has yet been reported. Unlike reactions in solution, free rotation of bonds in an enzyme active site may not occur because of specific binding interactions with groups or atoms in molecules. Such binding of the phenyl and amino groups in *trans*-2-phenyl(aminomethyl)cyclopropane would restrict rotation, thereby preventing proper orbital overlap with the α-amino radical. To test for this possibility,[10] (aminomethyl)cubane (**4**; Scheme III) was investigated as a potential inactivator of MAO. The rate constant for ring cleavage of the cubylcarbinyl radical is 3×10^{10} s^{-1}, greater than that of any other radical derived from a saturated hydrocarbon system to

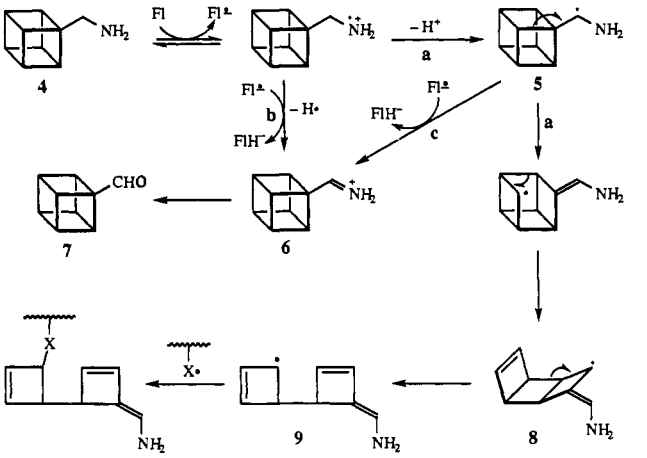
Scheme I



Scheme II



Scheme III



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date.[11] If an α-amino radical (**5**, pathway a) is generated in this case, there are, by symmetry, three possible bonds that can break; therefore, it is much more likely that proper orbital overlap will be achieved and, if an α-amino radical is formed, that ring cleavage and inactivation will occur. If, on the other hand, hydrogen atom transfer (pathway b) is the major pathway or if second electron transfer is competitive with cubane ring cleavage (pathway c), then cubanecarboxaldehyde (**7**) should be produced.

(Aminomethyl)cubane[12] was found to be a time-dependent irreversible inactivator of purified MAO-B. The kinetic constants for inactivation in 100 mM Tris–HCl buffer, pH 9.0 at 25 °C, determined by the method of Kitz and Wilson,[13] were K_I = 0.17 mM and k_{inact} = 0.033 min^{-1}. Dialysis (100 mM Tris–HCl buffer, pH 9.0) of the inactivated enzyme for 48 h with several buffer changes did not result in any return of enzyme activity. The substrate, 2-phenylethylamine, protected the enzyme from inactivation,[14] indicating that inactivation occurs at the active site. The presence of β-mercaptoethanol (2.3 mM) in the inactivation buffer did not have an effect on the rate of inactivation by 0.29 mM (aminomethyl)cubane. Therefore, an electrophilic species (either cationic or radical in nature) released from the enzyme is *not* responsible for the observed inactivation; inactivation must be occurring because of a reactive species generated at the active site prior to its release. (Aminomethyl)cubane also is a substrate for MAO-B;[15] K_m = 0.14 mM and k_{cat} = 5 min^{-1}. The partition ratio, as calculated from k_{cat}/k_{inact}, equals 152. After incubation of the enzyme (29 μM) with (aminomethyl)cubane (30 mM) in 4 mL of 200 mM sodium pyrophosphate buffer, pH 8.3, for 48 h (no enzyme activity remaining), the enzyme mixture was acidified to pH 1–2 with 10% HCl, extracted with methylene chloride (three times), washed with brine, dried (Na_2SO_4), concentrated with a stream of N_2, and then analyzed for products by GC/MS[16] and by 400-MHz NMR. Three metabolites were detected by GC: cubanecarboxaldehyde[17] (**7**) and two unknown metabolites; no cubanecarboxylic acid was detected.[18] Although we have not yet identified the structures of the unknown metabolites, GC–mass spectrometry (EI, 70 eV; mass range 30–550) indicated that they do not contain an intact cubane structure.[19] NMR analysis of the crude mixture showed the presence of cubanecarboxaldehyde as well as aromatic protons, which are suggestive of the formation of metabolites derived from ring-opened derivatives such as **8** or **9**. When the experiment was repeated, but with the time of incubation of the enzyme with (aminomethyl)cubane (18 mM) shortened to 2.5 h, the same result was obtained, except that a few additional minor metabolite peaks were observed and the peak ratios were different; the NMR spectrum also showed fewer aromatic resonances. These observations are consistent with the formation of unstable products derived from cleavage of the cubane structure.[10] Incubation of the enzyme with cubanecarboxaldehyde and cubanecarboxylic acid under the conditions of the experiment carried out with (aminomethyl)cubane did not give the products generated from MAO-catalyzed oxidation of (aminomethyl)cubane; cubanecarboxaldehyde was slowly oxidized to cubanecarboxylic acid, which was stable under these conditions. No other decomposition products from cubanecarboxaldehyde were detected. Therefore, the new products are not decomposition products of cubanecarboxaldehyde or cubanecarboxylic acid and may be related to either **8** or **9** (Scheme III).

These results support a mechanism involving one-electron transfer from the amine to the flavin[2,3] followed by removal of the α-proton to give an α-amino radical intermediate (Scheme III, pathway a; **5**). This radical, then, may partition between rapid cubane ring cleavage (pathway a) to give a reactive radical which can either become attached to the active site or be released into solution to form the observed products, or it may transfer a second electron (pathway c) to give the imine **6**, which leads to formation of **7**. The identities of the metabolites are being pursued via chemical model studies and enzyme labeling studies.

Acknowledgment. We are grateful to the National Institutes of Health (Grant GM32634 (R.B.S.)) and to the National Science Foundation (Grant CHEM-9010059 (P.E.E.)) for financial support of this research, to Elena Galoppini for a gift of cubanecarboxaldehyde,and to Nobuhiro Kanomata for donating a sample of cubanecarboxylic acid.

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(14) The $t_{1/2}$ for inactivation with 0.17 mM (aminomethyl)cubane was 35 min, and with 0.17 mM (aminomethyl)cubane plus 0.5 mM 2-phenethylamine was 52 min.

(15) Kinetic constants were determined by measuring the amount of hydrogen peroxide formed as previously described.[4b]

(16) The concentrated metabolite solutions were introduced into a VG70–250SE mass spectrometer via a HP 5890 GC equipped with a 30-m × 0.25-mm DB-17 capillary column. With this system, cubanecarboxaldehyde has a retention time of 8.17 min and the other metabolites have retention times of 9.23 and 12.35 min.

(17) An authentic sample of cubanecarboxaldehyde was synthesized by oxidation of ((*N*,*N*-diisopropylamino)methyl)cubane with dimethyldioxirane; compare with Chen, N.; Jones, M., Jr.; White, W. R.; Platz, M. S. *J. Am. Chem. Soc.* **1991**, *113*, 4981–4992.

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(19) One of the unknown metabolites had no *m*/*z* 103 peak, a major fragment for monosubstituted cubanes, and, therefore, is not a cubane derivative. The other metabolite had a *m*/*z* 103 peak, but the base peak was *m*/*z* 105. This compound, however, does not have the expected stability of a cubane derivative; upon standing in chloroform for less than 48 h, it decomposed completely.