Radical Ideas about Monoamine Oxidase

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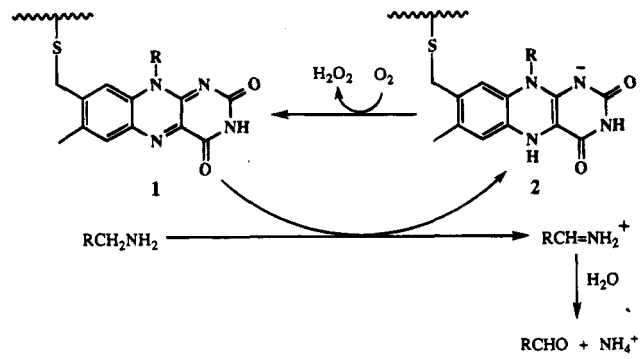
Monoamine oxidase (MAO) catalyzes the oxidation of a variety of amine neurotransmitters, such as norepinephrine, serotonin, and dopamine. It belongs to a family of enzymes known as flavoenzymes because of their essential requirement for a flavin coenzyme.[1] In the case of MAO the flavin is covalently attached at the 8α-position to an enzyme active site cysteine residue (**1**), and it catalyzes the anaerobic conversion of amine substrates to the corresponding imines,[2] which are released from the enzyme and hydrolyzed to the corresponding aldehydes[3] (Scheme 1). The enzyme is inactive in the reduced flavin form (**2**) and requires molecular oxygen to oxidize it back to the native form. It was shown in 1968 that MAO exists in two different forms (called isozymes), referred to as MAO A and MAO B.[4] Although these isozymes have somewhat different compositions,[5] they catalyze the same reaction, only preferentially on different amine substrates. MAO A has been shown to be responsible for certain types of depression which arise from a decrease in the concentration of brain norepinephrine and/or serotonin,[6] and MAO B is linked to Parkinson's disease as a result of its degradation of brain dopamine.[7] Therefore, compounds that selectively inhibit or inactivate (slow down or prevent enzyme catalysis of) one of the MAO isozymes have been shown to be useful in the treatment of depression[8] or Parkinson's disease.[9] The elucidation of the mechanism of MAO would aid in the design of potential new antidepressant and antiparkinsonian drugs.

One of the approaches to the study of enzyme mechanisms that we have been using involves investigating the mechanisms of inactivation of MAO by a class of inactivators known as mechanism-based inactivators.[10] A mechanism-based enzyme inactivator is an unreactive compound that has a structural similarity to the substrate or product for the target enzyme. It is converted into a product or intermediate that is generally (but not always) reactive, and this causes inactivation of the enzyme, typically by covalent bond formation. Because these compounds are substrates for the enzyme, their conversion into the activated form proceeds, at least initially, by the normal catalytic mechanism. Therefore, any mechanistic information gleaned from the inactivation mechanism is directly related to the catalytic mechanism.

Richard B. Silverman was born on May 12, 1946, in Philadelphia. After having earned a B.S. degree in chemistry from The Pennsylvania State University in 1968, he entered Harvard University for graduate studies, where he joined the research group of E. J. Corey. At the end of the first semester Uncle Sam called, and he spent two years in the U.S. Army, during which time he received an Army Commendation Medal. Upon his return to Harvard, he joined the research group of David Dolphin and in 1974 received his Ph.D. After two years as a postdoctoral associate with Robert Abeles in the Graduate Department of Biochemistry at Brandeis University, he joined the faculty at Northwestern University, where he currently is professor of chemistry and professor of biochemistry, molecular biology, and cell biology. He also is a member of the Northwestern University Institute for Neuroscience. His research interests are in the design, synthesis, and mechanism of enzyme inactivators and in the study of enzyme mechanisms.

Scheme 1



Proposed Mechanism

MAO is a very promiscuous enzyme, catalyzing the oxidation of a variety of primary, secondary, and tertiary alkyl and arylalkyl amines,[11] although the preference is for primary amines. Stereospecific removal of the *pro-R* hydrogen has been demonstrated for several different substrates.[12] When we became interested in this enzyme in the late 1970s, little was known about the mechanism of oxidation of amines by MAO. It had been known by then, however, that amines could be oxidized nonenzymatically by chemical oxidizing agents,[13] electrochemically,[14] and photo-

(1) A coenzyme is an organic molecule or transition metal ion that is essential for the enzyme activity.

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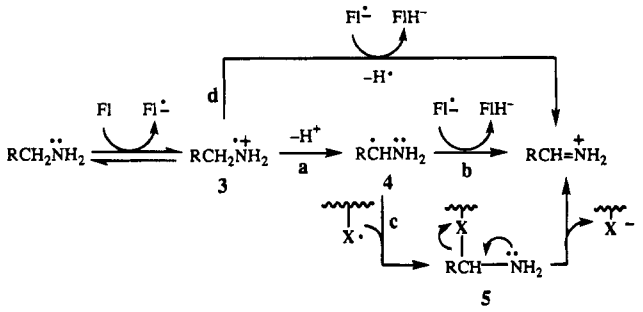
Scheme 2



chemically.[15] On the basis of these nonenzymatic processes, which all occur by one-electron mechanisms, it was proposed by my group[16] and by the Krantz group[17] that oxidations catalyzed by MAO also should proceed by a one-electron mechanism (Scheme 2 shows an expanded version of the original mechanism proposed). One-electron transfer from the substrate amino group to the oxidized flavin (Fl) gives the amine radical cation (**3**) and the flavin semiquinone ($Fl^{\bullet -}$). Loss of a proton (pathway a) gives the α-amino radical (**4**), which can either transfer the second electron to the flavin semiquinone to give reduced flavin (FlH^-, pathway b) or undergo radical combination with an active site radical (pathway c) to give a covalent adduct (**5**), which should decompose by β-elimination to give the iminium ion. A potential alternative to proton transfer (pathway a) followed by electron transfer (pathway b) is hydrogen atom transfer (pathway d), which bypasses the α-amino radical intermediate (**4**). The X group in Scheme 2 could be either the flavin semiquinone generated in the first step or an amino acid radical formed by hydrogen atom transfer from the amino acid to the flavin semiquinone. Removal of a proton from **3** should be a relatively facile process for MAO because the pK_a of a hydrogen adjacent to a tertiary amine radical cation is about 10,[18] and the pH optimum of the enzyme is 9. As a rationalization for the observed primary kinetic isotope effect on MAO-catalyzed oxidation of α,α-$^2H_2$-labeled substrates,[19] Krantz and co-workers[17] suggested that the first electron transfer is reversible and, therefore, not rate determining. Depending upon the relative magnitudes of the rate constant for the reverse of the electron transfer and that for the proton abstraction step, the kinetic isotope effect could range from 1 to 7. These kinds of isotope effect variations have been observed in the case of cytochrome P-450 catalyzed reactions.[20]

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Rationalizations for Electron Transfer

One aspect of the mechanism shown in Scheme 2 that requires rationalization is the initial one-electron transfer from the amine to the flavin. This is an apparent highly thermodynamically uphill process in solution; the peak oxidation potential for a primary amine is nearly +1.5 V vs the standard calomel electrode (the onset of oxidation, however, occurs at much lower potentials), and the reduction potential for free riboflavin is −0.25 V, although flavoprotein redox potentials have been determined in the −0.49 to +0.19 V range.[21] However, irreversible redox processes do not correlate with standard redox potentials.[22] Furthermore, this large apparent energy barrier to electron transfer may not be relevant to the reaction catalyzed at the active site of an enzyme. Intrinsic binding,[23] which could distort the bonds of the amine and the flavin, would lower the redox potentials of the amine and the flavin. Distortion of the amine bonds would result in the lowering of the energy required to remove a nonbonded electron from the nitrogen orbital, thereby lowering the oxidation potential of the substrate amine.[24] The oxidized form of flavin is planar and highly conjugated, but reduced flavin is bent to avoid antiaromaticity.[25] Therefore, distortion of the flavin rings by the enzyme to favor binding of a bent (reduced) form would result in the lowering of the LUMO energy of the flavin and make electron transfer to the enzyme-bound flavin a more facile process. This enzyme-induced distortion lowers the activation energy for electron transfer by destabilization of the oxidized form (ground state energy increase) and by stabilization of the reduced form (decrease of transition state energy). A conformational change in the enzyme as a result of substrate binding could lead to such a distortion. In fact, it has been demonstrated that the flavin E_0 in MAO A and B increases by about 500 mV by addition of a poor substrate, and it was predicted that the change in the redox potential should be even greater if it could be measured in the presence of a good substrate.[26a] Recently, a model receptor was used to demonstrate that flavin radical anions are stabilized by hydrogen bonding, thereby lowering the flavin reduction potential by an additional 155 mV.[26b] The transition state energy also would be lowered by approximation,[27] the proximity effect of the enzyme binding the substrate close to the flavin cofactor, a common mechanism for catalysis by enzymes in any bond-breaking reaction. Solvent also has a large effect on oxidation potentials. The $E_{1/2}$ of an organic compound in an irreversible electrochemical reaction can change by 0.5 V just by changing the molar ratio of the organic solvent to

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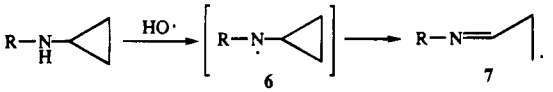
Scheme 3

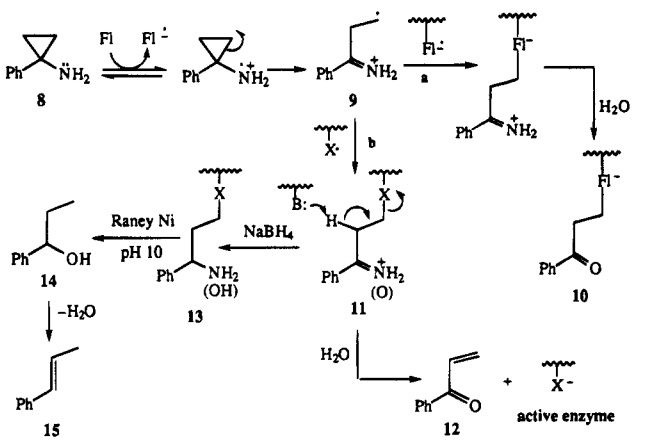


water.[28] MAO has a highly hydrophobic active site to accommodate the hydrophobic substrates on which it catalyzes oxidation reactions. The degree of hydrophobicity at the active site could be optimized for greatest lowering of the oxidation potential of the substrate. When medium effects on the redox potential[29] and the problems of comparing differing reference electrode potentials between solvents[30] are taken into account, there may not be the magnitude of difference in the redox potentials that there appears to be from solution data.

Mechanistic Approach

When we became interested in MAO, little was known about the catalytic mechanism of the enzyme, and there was no evidence for radical intermediates. Tan et al.[31] found no evidence for radicals by EPR spectroscopy during substrate turnover; stopped-flow kinetics experiments failed to provide evidence for a flavin semiquinone intermediate.[32] Therefore, if radical intermediates are formed, they must be very short-lived, low in concentration, or spin paired with another radical.[17] Because of the lack of spectral evidence for radical intermediates, we took the following chemical approach to the detection of a radical intermediate. If substituent reporter groups that could divert the chemistry upon formation of a radical intermediate were attached to substrates for MAO, then the mechanism could be inferred by product analysis. The first reporter group selected was the cyclopropyl group. The selection of this group was based on the laser flash photolysis work of Maeda and Ingold,[33] who found that secondary aminyl radicals could be generated and observed by low-temperature EPR spectroscopy, but when the corresponding cyclopropylaminyl radical (**6**, Scheme 3) was generated by the same method, it could not be observed; instead, the ring-cleaved primary radical product (**7**) was detected. The rate of cyclopropyl ring opening was too fast to measure and was estimated at $>5 \times 10^8$ s^{-1}. This diversion of the normal chemistry at a rate competitive with enzymatic rates was seen as a potential approach to provide evidence for the formation of an aminyl radical (or amine radical cation, depending upon the pH) during substrate turnover. Consequently, a variety of cyclopropylamine-containing substrate analogues were synthesized as potential mechanism-based inactivators[34] and were studied as substrates for MAO.[35-44] Upon

Scheme 4



incubation of MAO with each of these compounds, the enzyme became inactivated (irreversible loss of enzyme activity), as would be expected for oxidation to the aminyl radical (cation) followed by cyclopropyl ring cleavage and attachment to the enzyme.

Example of the Mechanistic Approach To Detect Initial Electron Transfer

As an example of the approach taken, consider the inactivation of MAO by 1-phenylcyclopropylamine (**8**, Scheme 4). Because of the low concentration of enzyme, it is necessary to synthesize radioactively-labeled analogues to be able to detect the metabolites and to determine if and how much of the compound becomes covalently attached to the enzyme. With the use of [*phenyl*-^{14}C]-**8** it was shown that this compound becomes attached to MAO in a 1:1 stoichiometry either at the flavin or at an active site cysteine residue;[43] the product of attachment at the flavin was stable, but the cysteine residue adduct was not. On the basis of the mechanism shown in Scheme 2 inactivation could arise from the activation of the cyclopropyl ring by the proposed normal catalytic mechanism to give a reactive primary radical (**9**), which could react either with the flavin semiquinone (pathway a), leading to adduct **10**, or with an amino acid radical (pathway b) to give **11**. Retro-Michael reaction would cleave the inactivator from the enzyme to yield acrylophenone (**12**) and active enzyme. The structure of **10** was determined by carrying out three aqueous-based organic reactions on it. Treatment with sodium boro-[^{3}H]hydride led to the incorporation of 1 equiv of tritium into the inactivated enzyme that did not go into native enzyme; this is consistent with the presence of a ketone (or imine) moiety. Baeyer–Villiger oxidation with trifluoroperoxyacetic acid (to give the phenyl ester) followed by saponification with sodium hydroxide released all of the radioactivity from the

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enzyme as [^{14}C]phenol; this is consistent with a phenyl ketone moiety. Finally, incubation of labeled enzyme with 0.5 M sodium hydroxide released the radioactivity as acrylophenone; this is consistent with enolization of **10** followed by β-elimination of the flavin. Attachment to the flavin was demonstrated by first showing spectrophotometrically that the flavin was in a reduced state and then carrying out a proteolytic digestion of the labeled enzyme and showing that by gel filtration chromatography the radioactivity comigrated with the flavin absorption.

The adduct to the amino acid was stable enough ($t_{1/2}$ of about 1 h) for isolation and determination of the kinetics of decomposition to **12** and active enzyme. The amino acid adduct was stabilized by treatment with sodium borohydride, which, presumably, reduced the ketone and prevented enolization and β-elimination (**13**). The flavin spectrum indicated that it was oxidized, suggesting that this adduct was not attached to the flavin. The amino acid radical could arise from hydrogen atom abstraction by the flavin semiquinone. If that is the case, then the active site amino acid residue must be a good hydrogen atom donor and, to accommodate a retro-Michael cleavage, must be a good leaving group. The two amino acids that possess those properties are cysteine (**11**, X = S) and tyrosine (**11**, X = O). These were diffentiated by treatment of the radioactively-labeled and sodium borohydride reduced amino acid adduct (**13**) with Raney nickel, a reagent known to reduce C–S bonds exclusively;[45] in proteins, Raney nickel was shown to reduce only cysteine and methionine residues.[46] The only radioactive product isolated from Raney nickel treatment was *trans*-β-methylstyrene (**15**, Scheme 4), which was shown in a control reaction to be the product when 1-phenylpropanol (**14**), the expected product of Raney nickel reduction of a cysteine adduct, was incubated under the conditions of the experiment. To confirm this result, the labeled enzyme was shown by 5,5′-dithiobis(2-nitrobenzoic acid) titration to contain only five cysteine residues, whereas the native enzyme titrated for six. Therefore, one cysteine is lost upon amino acid labeling. The proposed electron transfer mechanism shown in Scheme 4 was supported by a photochemical study by Kim et al.,[47] who used a model flavin as the electron acceptor and showed that single electron transfer chemistry produces the same flavin adduct (**10**) as is produced in the MAO study.

Studies similar to those described above for 1-phenylcyclopropylamine were carried out for many of the other cyclopropylamine analogues, and the results can be interpreted with a similar mechanism. The principal difference in the various cyclopropylamines was in their site of attachment to the enzyme; in some cases, attachment was exclusively to the flavin;[37] in others attachment was only to an amino acid;[36,41] and some cases showed attachment to either the flavin or the amino acid.[39,40,43] The fact that all of these cyclopropylamines inactivate MAO and result in cyclopropyl ring opened adducts is consistent with the intermediacy of a cyclopropylaminyl radical.

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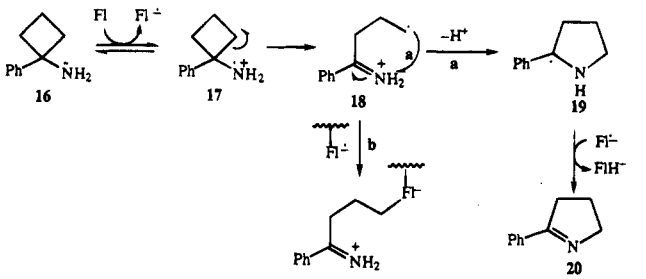
Scheme 5



Another Mechanistic Approach To Detect Initial Electron Transfer

Another approach taken to support an initial one-electron oxidation was the design of a compound that would undergo a known one-electron rearrangement upon one-electron oxidation. The compound chosen was 1-phenylcyclobutylamine (**16**, Scheme 5). MAO-catalyzed one-electron oxidation would generate **17**, which would trigger cyclobutyl ring cleavage,[33] leading to **18**. This intermediate has a built-in radical trap, i.e., the imine double bond. There is ample precedence that intermediates related to **18**, regardless of whether they contain a carbon–carbon,[48] carbon–oxygen,[49] or carbon–nitrogen[50] double bond, undergo *endo*-cyclization to cyclopentyl, tetrahydrofuranyl, or pyrrolidinyl radicals, respectively. Second-electron oxidation would give the corresponding cyclopentene, dihydrofuran, or pyrroline, respectively. Incubation of MAO with 1-phenylcyclobutylamine led to time-dependent inactivation, presumably by the trapping of **18** by the flavin semiquinone (Scheme 5, pathway b).[51] When aliquots were removed from the incubation mixture periodically and analyzed by HPLC and mass spectrometry, a time-dependent decrease in the concentration of 1-phenylcyclobutylamine was observed with a concomitant increase in the formation of 2-phenyl-1-pyrroline (**20**, Scheme 5, pathway a). These results are consistent with initial formation of the 1-phenylcyclobutylaminyl radical. This same series of experiments was carried out using cytochrome P-450, an enzyme for which there is a large body of evidence to support radical intermediates,[52] and the same rearrangement product was obtained as with MAO.[53] The observation of a known radical-induced rearrangement catalyzed by MAO and by cytochrome P-450 supports a radical mechanism for MAO.

Intermediate **19** (Scheme 5) should be more long-lived than an aminyl radical, such as **17**, or a simple carbon radical, such as **18**. If it is stable enough to leak out of the active site, it should be possible to trap it with a radical spin trap. Incubation of MAO with either 1-benzoylcyclobutylamine (**16** except Bz substituted for Ph) or 1-phenylcyclobutylamine (**16**) in the presence of the radical spin trap α-phenyl *N*-*tert*-butylnitrone resulted in a time-dependent formation

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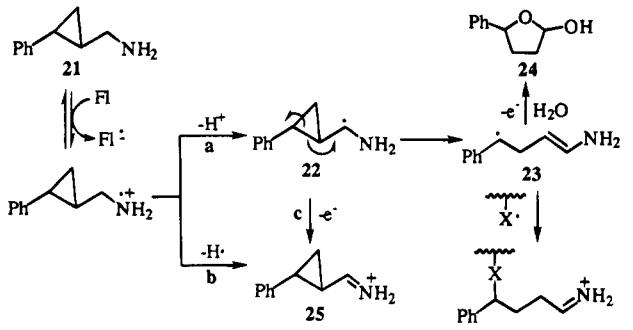
Scheme 6



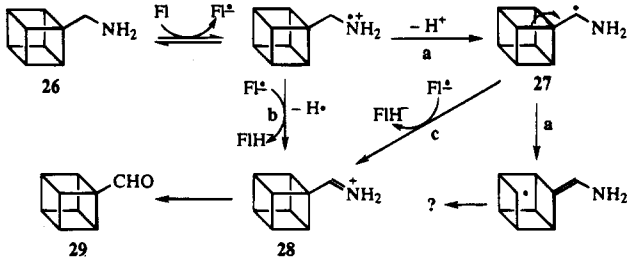
and increase in an EPR triplet of doublets centered at a g value of 2.006, suggestive of the formation of a stable nitroxyl radical.[54]

Differentiation of Proton/Electron and Hydrogen Atom Transfer Mechanisms

All of the results described above are consistent with initial electron transfer to generate an aminyl radical (cation). We then sought to differentiate a proton/second electron transfer mechanism (Scheme 2, pathway a) from a hydrogen atom mechanism (Scheme 2, pathway d). The principal difference in these pathways is the presence or absence of intermediate **4**. A related approach was taken to differentiate these pathways as was taken to detect the aminyl radical. The *trans*-(2-phenylcyclopropyl)carbinyl radical is one of the most unstable radicals, decomposing, with cleavage of the cyclopropyl ring, at a rate of 3×10^{11} s^{-1}.[55] *trans*-2-Phenyl-1-methylcyclopropane was used successfully to probe for the intermediacy of the *trans*-(2-phenylcyclopropyl)carbinyl radical during hydroxylation by a non-heme iron monooxygenase.[56] It was thought that a similar approach, namely, incubation of MAO with *trans*-1-(aminomethyl)-2-phenyl cyclopropane (**21**, Scheme 6), would reveal the relevant pathway. If proton removal occurred following initial electron transfer, the intermediate cyclopropylcarbinyl radical (**22**, Scheme 6, pathway a) would result. Ring cleavage would then give **23**. If hydrogen atom transfer occurred, however, the cyclopropylcarbinyl radical would be bypassed and the corresponding cyclopropyl imine would form (**25**, Scheme 6, pathway b). It was unclear what product would be expected if **22** were generated, so a chemical model study was carried out to determine the potential fate of **22**.[57] Treatment of 2-(*trans*-2-phenylcyclopropyl)glycine with peroxydisulfate ion and a catalytic amount of silver(I) ion in pH 7.0 buffer at 40 °C produced 2-hydroxy-5-phenyltetrahydrofuran (**24**).

Incubation of MAO with **21** produced *trans*-2-phenylcyclopropanecarboxaldehyde exclusively; no inactivation occurred, and no **24** was produced. This meant that either the exclusive pathway for MAO-catalyzed oxidation of amines was pathway b (Scheme 6) or second-electron transfer (pathway c) occurs faster than cyclopropyl ring cleavage. Two rationalizations for why second-electron transfer could be faster than ring

Scheme 7



cleavage are (1) that the amino group stabilizes the radical, thereby slowing down the rate of ring cleavage, and (2) that free rotation is frozen during enzyme binding so that overlap between the α-carbon radical orbital and the orbitals of the cyclopropyl ring is poor. It is known that heteroatom stabilization of radicals can be considerable,[58] but an exact effect of the amino group on the rate constant for cyclopropyl ring cleavage is not known.

To test the orbital overlap hypothesis, (aminomethyl)cubane (**26**, Scheme 7) was synthesized.[59] If an α-radical is generated during turnover (**27**, pathway a), then there are three symmetrical bonds (boldfaced in **27**) in three different orientations that could overlap with the orbital containing the radical, any of which would lead to cubane ring cleavage at a rate of 3×10^{10} s^{-1}.[60] Treatment of MAO with (aminomethyl)cubane led to time-dependent irreversible inactivation of the enzyme. Two metabolites were isolated; one was identified by NMR and GC–mass spectrometry as cubanecarboxaldehyde (**29**), and the other was shown by GC–mass spectrometry to be missing an intact cubane structure and to contain a resonance in the NMR spectrum in the aromatic region. The important finding is that the cubane structure is destroyed, indicative of the generation of an α-radical. The ring cleavage product could not have come from the corresponding cation (iminium ion **28**), because cubylcarbinyl cations are well-known to undergo exclusive rearrangement to homocubanes without ring cleavage,[61] and no evidence for a homocubane was found. Therefore, the decomposition of the cubane nucleus must result from generation of an α-radical or possibly from an α-carbanion.[62] Formation of cubanecarboxaldehyde could arise by second-electron transfer from the α-radical prior to cubane ring cleavage (pathway c). These results strongly argue against a transfer of a hydrogen atom from the amine radical (cation) (pathway b) and support the proton/electron transfer mechanism (pathway a).

Since cubane destruction could arise from generation of α-carbanion character, experiments were de-

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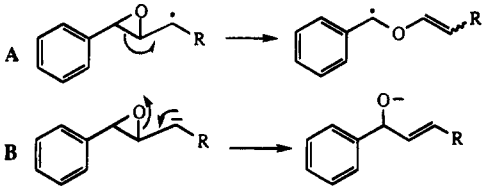
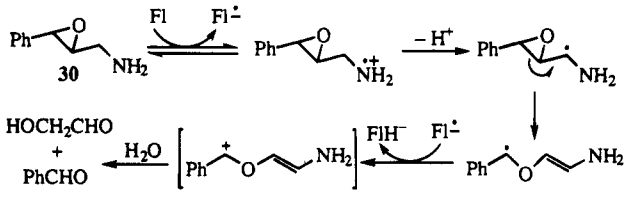
(62) The evidence for decomposition of cubanes by an α-carbanion is the reaction of a (cyanomethyl)- or ((methoxycarbonyl)methyl)cubane with LDA.[63] However, it is not clear that LDA reactions proceed by a direct deprotonation mechanism or by electron transfer mechanisms.[64]
(63) Klunder, A. J. H.; Zwanenburg, B. *Tetrahedron* **1975**, *31*, 1419–1426.
(64) Ashby, E. C.; Park, B.; Patil, G. S.; Gadru, K.; Gurumurthy, R. *J. Org. Chem.* **1993**, *58*, 424–437.

Scheme 8



Scheme 9

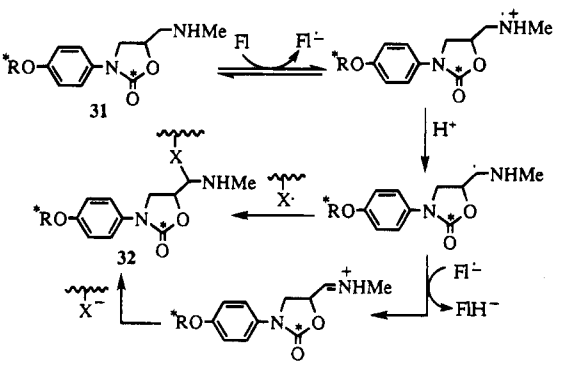


signed to differentiate a radical from a carbanion intermediate. It had been reported that 1-substituted 2,3-epoxy-3-phenylpropyl radicals cleave the C–C bond of the epoxide exclusively (Scheme 8A), whereas the corresponding carbanion leads to exclusive epoxide C–O bond cleavage (Scheme 8B).[65] Consequently, cinnamylamine 2,3-oxide (**30**, Scheme 9) was designed as a mechanistic probe for a radical versus a carbanion intermediate in MAO-catalyzed reactions.[66] Carbon–carbon bond cleavage of cinnamylamine 2,3-oxide α-radical followed by second-electron oxidation and hydrolysis would give benzaldehyde and glycolaldehyde, whereas C–O bond cleavage by the α-carbanion would lead to cinnamaldehyde as the product. No cinnamaldehyde was detected, only benzaldehyde and glycolaldehyde,[66] providing evidence against carbanion mechanisms and support for the formation of an α-radical during MAO-catalyzed reactions. This result coupled with the results described above with the cubane analogue provides very strong support for the formation of an α-radical during MAO processing of primary amines.

Evidence for a Covalent Pathway

From an α-radical intermediate (**4**, Scheme 2) either second-electron transfer to the flavin semiquinone (pathway b) or radical combination to **5** followed by β-elimination (pathway c) is reasonable. Data to support pathway c, at least for some substrates, has been obtained. 3-Aryl-5-((methylamino)methyl)-2-oxazolidinones were reported in the early 1980s to be time-dependent inactivators of MAO B.[67] We found that inactivation of MAO B by either (*R*)- or (*S*)-[^{3}H]-3-aryl-5-((methylamino)methyl)-2-oxazolidinone (**31**, Scheme 10) led to the incorporation of 1 equiv of tritium per enzyme after denaturation, suggesting that a covalent adduct was formed.[68] (*R*)- and (*S*)-[*carbonyl*-^{14}C]-3-aryl-5-((methylamino)methyl)-2-oxazolidinone (**31**) also inactivated MAO B with the incorporation of 1 equiv of radioactivity after denaturation. In addition to labeled enzyme, 4 or 5 equiv

Scheme 10



of $^{14}CO_2$ was generated during inactivation by the (*R*) and (*S*) isomers, respectively. Loss of $^{14}CO_2$ comes from a reaction not on the pathway to inactivation which may involve radical-induced decarboxylation of the oxazolidinone.[69] The fact that both the tritium in the side chain and the ^{14}C in the oxazolidinone ring become attached stoichiometrically to the enzyme suggests that the entire molecule is part of the enzyme adduct (**32**, Scheme 10).

Why should this adduct, an analogue of **5** (Scheme 2, R = oxazolidinonyl), be stable whereas the proposed corresponding adducts with normal substrates of the enzyme (**5**, R = alkyl or arylalkyl) decompose readily by β-elimination to give the imine products? The answer may lie in the oxazolidinone ring. Adduct **32** (Scheme 10) has two heteroatoms (the methylamino group nitrogen and the X group of the enzyme) attached to an sp^3 carbon atom of the inactivator. These types of systems (e.g., acetals, aminals, thioaminals, etc.) are stabilized by electron-withdrawing groups attached to the α-position. In this case the electron-withdrawing atoms of the oxazolidinone could act to stabilize the adduct relative to groups such as alkyl and arylalkyl that are part of normal substrates for the enzyme. If the O and N atoms of the oxazolidinone are the cause for the stabilization effect, then the corresponding lactone (replacement of the N by C–H) also should inactivate the enzyme. In fact, both the *cis*- and *trans*-lactones were found to inactivate MAO B.[70] Next the corresponding lactam (replacement of O in the oxazolidinone by CH_2) was tested, and it too inactivated the enzyme.[71]

Whereas the oxazolidinone and lactone have electron-withdrawing groups at the α-carbon, the lactam has its electron-withdrawing groups (the carbonyl and the N) at the β-carbon. Electron-withdrawing effects diminish greatly with distance; at the α-position, the effect can be large, but at the β-position, it is generally thought to be weak. Therefore, it was curious that the lactam also inactivated the enzyme. To gain support for the inductive effect hypothesis for adduct stabilization, a chemical model study was carried out.[72] On the assumption that the rate of breakdown of the enzyme adduct would mimic the rate of acid-catalyzed hydrolysis of acetals, a series of acetals of aldehydes containing various substituents designed to mimic the enzyme adducts of good substrates and of

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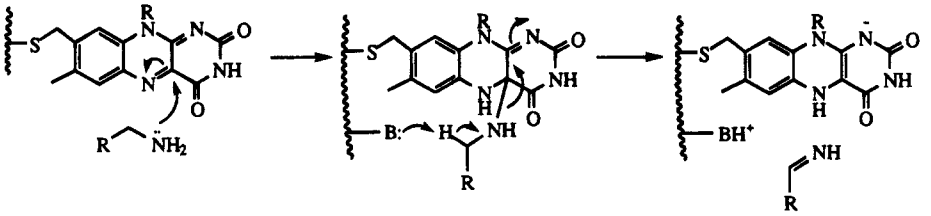
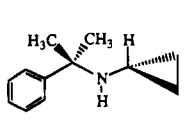
Scheme 11



inactivators was synthesized. Models for adducts with good substrates ($RCH(OEt)_2$, where R = Ph, cyclopentyl, 3-arylcyclopentyl, and Bn) undergo rapid hydrolysis. The rate constant for hydrolysis of the model for the lactam inactivator (R = 3-arylpyrrolidinonyl), however, is 167 times smaller than that for the corresponding 3-arylcyclopentyl compound; the rate constant for decomposition of the lactone is 7700 times smaller, and that for the oxazolidinone is 238 000 times smaller. This is a remarkable inductive effect and appears to be sufficient to stabilize the inactivator adduct. It is not clear if the formation of this stable adduct represents the blockage of the normal catalytic mechanism or is an alternative pathway for molecules that have a higher second-electron oxidation potential.

Evidence against a Nucleophilic (Polar) Mechanism

Recently, a previously proposed mechanism for MAO[73] was revived by Mariano and co-workers[74,75] in the form of two chemical model studies. Some of our evidence against such a mechanism[66,76] is outlined here. In the first set of experiments Mariano and coworkers demonstrated that, under certain drastic conditions (wet acetonitrile containing 10 mM HCl heated to 80 °C for 7 days to give a 30% yield of oxidized primary amine), benzylamine could be oxidized to benzaldehyde in the presence of the model flavin 3-methyllumiflavin.[74] This reaction was shown to be best (by far) with a primary amine and not to proceed at all with tertiary amines, suggesting a nucleophilic mechanism (Scheme 11). However, this reaction appears to be irrelevant to the mechanism of MAO, because MAO catalyzes the oxidation of secondary and tertiary amines as well as primary amines (and, in many cases, at a faster rate).[77] Furthermore, steric hindrance does not appear to be a problem in MAO-catalyzed amine oxidations, because several very sterically-hindered secondary and tertiary amines, e.g., **33** and **34**, are oxidized by MAO;[76] these compounds would not be able to undergo a nucleophilic mechanism. Also, as described above, we have carried



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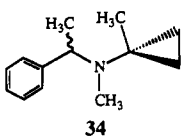
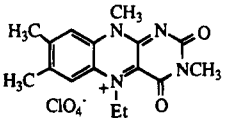


34

out the oxidation of cinnamylamine 2,3-oxide (Scheme 9)[66] and have shown that no products of an α-carbanion intermediate (C–O bond cleavage) were observed, only products of an α-radical intermediate (C–C bond cleavage). The nucleophilic mechanism could generate α-carbanionic character and should lead to C–O bond cleavage products; it is not consistent[65] with the observed C–C bond cleavage.[66] Since a primary amine substrate (**30**) was used in this study, the MAO results described here indicate that this chemistry could be representative of MAO-catalyzed oxidation of normal primary amine substrates.

In addition to all of the evidence presented above supporting a radical mechanism, we also have shown that the oxidation of (*R*)- and (*S*)-1-phenyl[2,2-$^2H_2$]-cyclopropylamine proceeds with a normal secondary deuterium isotope effect on ring opening.[44] This is consistent with a mechanism involving ring cleavage followed by radical combination. A nucleophilic mechanism (Scheme 11) would involve attack of the active site amino acid on the cyclopropyl ring concomitant with elimination of the reduced flavin; this mechanism would result in an inverse secondary isotope effect, which is not observed.

The second model study by Mariano and co-workers[75] demonstrates that with the use of 3-methyl-5-ethyllumiflavinium perchlorate (**35**) it is possible to show that primary amines, such as benzylamine,[74] cyclopropylamines, α-silyl amines, and hydrazines, can undergo nucleophilic mechanisms. However, **35** is a drastically altered flavin, one which strongly favors nucleophilic addition reactions; consequently, it is not surprising that the nucleophilic addition products are observed. This is akin to the numerous earlier studies in which 5-deazaflavin (**36**) was substituted for flavin both in nonenzymatic studies and in enzymatic studies (but not with MAO, because the flavin in MAO is covalently bound to the enzyme). Enzymatic oxida-



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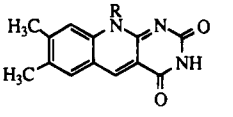

36

tions similar to those with flavin were observed. However, it was later shown that 5-deazaflavin strongly favors two-electron chemistry (not radical chemistry) and, therefore, is *not* an appropriate model for flavin-dependent enzymes; rather, it is more appropriate as a model for reactions catalyzed by pyridine nucleotide (NAD^+/NADH)-dependent enzymes.[78,79] "Flavin model" **35** also appears to be a dubious chemical model for flavin-dependent enzymes. However, there is now

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substantial evidence that quinoproteins, such as tryptophan tryptophylquinone-dependent[80] and topa-dependent[81] enzymes, catalyze a nucleophilic oxidation mechanism on primary amines to give the same products as would MAO. Those enzymes oxidize primary amines, but not tertiary amines. I would contend that **35** is probably a good model for quinoproteins rather than for flavoenzymes.

Mariano and co-workers[74,75] suggest that their model for nucleophilic chemistry may be important only in the oxidation of primary amines, not tertiary amines. However, many of the studies cited in this Account were carried out with primary amines, and they support radical mechanisms. Conversely, the enzyme chemistry that we observe for the primary cyclopropylamines and α-silyl amine is the same as we observe for the corresponding tertiary amines; the model studies are only applicable to the primary amine reactions and, therefore, are not consistent with the MAO results.

Evidence against a Direct Hydrogen Atom Abstraction Mechanism

On the basis of the above discussion, it would appear that the most reasonable mechanism for MAO-catalyzed oxidation of amines is pathway a/b or a/c (Scheme 2). An alternative radical mechanism, however, has been proposed. A large deuterium isotope effect on the MAO-catalyzed oxidation of a substrate analogue (called MPTP) as compared with a very low isotope effect on the electrochemical oxidation of this same analogue was used as evidence for a direct hydrogen atom abstraction mechanism.[82] Dinnocenzo and Banach[18] caution against these types of comparisons because the observed isotope effects are clearly a function of the structure of the cation radical, the base, and the surrounding medium. Also, enzyme-catalyzed reactions can be very different from solution reactions, particularly when carried out under such different conditions and especially if multiple steps are involved. The rates of each step can be different in solution and in the enzyme, and the pK_a of the acceptor will most likely be different. When the pK_a of the proton acceptor is matched with the acidity of the proton being removed, then maximal isotope effects are observed.[83] It is not at all surprising, then, that there would be a large difference between isotope effects obtained in a model study and with an enzyme.

Recently, Walker and Edmondson[84] reported a failure to observe the formation of flavin radical intermediates spectroscopically, and from stopped flow kinetics and kinetic isotope effect studies of substituted benzylamines, they concluded that an initial single-electron transfer mechanism was unlikely. They also cite the observation that *N,N*-dimethylbenzylamine is not a substrate for MAO, whereas benzylamine is, suggesting that a tertiary amine is less susceptible to oxidation than a primary amine. However, most other *N,N*-dimethylamines are oxidized[85] and many other tertiary amines are substrates;[77] it is not clear why *N,N*-dimethylbenzylamine is an anamoly, but it may have to do with an incorrect alignment of nonbonded electrons on the amine with the flavin. On the basis of competitive k_H/k_T and k_D/k_T kinetic isotope effect studies on *p*-methoxybenzylamine as a function of temperature, it was concluded[86] that hydrogen tunneling is important in the hydrogen abstraction step of the MAO-catalyzed oxidation of this substrate. In both cases, it was suggested that a mechanism involving direct hydrogen atom abstraction from the substrate to give the α-radical was more consistent with the kinetic results than was electron transfer/proton transfer, although it is not clear what radical species at the active site would be involved in a direct hydrogen atom abstraction. Furthermore, this conclusion is inconsistent with all of the studies of MAO-catalyzed oxidations of the cyclopropylamines[11d,16,35-44,54,57] and a cyclobutylamine.[51] In some of these examples α-hydrogen atom abstraction is precluded, because there is no α-hydrogen atom, and several of the flavin adducts that were isolated could not arise from direct cyclopropyl or cyclobutyl cleavage by oxidized flavin. None of the results of these reactions can be rationalized by a hydrogen atom mechanism.

Concluding Remarks

Although there are certain experiments that leave doubt regarding the catalytic mechanism of MAO, the overwhelming amount of data seems to suggest that a radical mechanism is most consistent. Interpretations of kinetics and spectroscopic results are subject to much speculation and are dependent upon making a variety of assumptions, including the assumption that the lifetime of an unobserved intermediate would be sufficiently long for observation. The observed isotope effect on α-C−H bond cleavage of benzylamine analogues can arise from a reversible SET step in which the back reaction is very fast; therefore, there may be no accumulation of amine radical cation or flavin semiquinone species for spectroscopic detection.[87] Isolation and structure identification of reaction products can be verified by direct chemical analysis. Product analysis is not necessarily a definitive approach for the establishment of a mechanism, but it is generally very useful to eliminate certain possible mechanisms from consideration.

The author is fortunate to have had so many capable coworkers over the years. He expresses his sincere thanks to them for their contributions to this research. He also acknowledges the National Institutes of Health (Grant GM32634) for financial support and thanks Professors Grissom and Edmondson for a preprint of their manuscript on rapid scan stopped-flow and magnetic field studies of monoamine oxidase B.

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(87) Recently,[88] studies were brought to my attention in which no flavin semiquinone intermediate could be detected by rapid scan stopped-flow spectrophotometry and no magnetic field dependence was observed during catalytic turnover. However, if the first electron transfer is reversible and the back reaction is much faster than the foward electron transfer, the lifetime for the flavin semiquinone could be less than 1 ms (the limit of detection).
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