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Review

Questions in the Chemical Enzymology of MAO

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Abstract: We have structure, a wealth of kinetic data, thousands of chemical ligands and clinical information for the effects of a range of drugs on monoamine oxidase activity in vivo. We have comparative information from various species and mutations on kinetics and effects of inhibition. Nevertheless, there are what seem like simple questions still to be answered. This article presents a brief summary of existing experimental evidence the background and poses questions that remain intriguing for chemists and biochemists researching the chemical enzymology of and drug design for monoamine oxidases (FAD-containing EC 4.1.3.4).

Keywords: chemical mechanism; kinetic mechanism; oxidation; protein flexibility; cysteine modification; reversible/irreversible inhibition; molecular dynamics; simulation

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1. Introduction

Monoamine oxidase (E.C. 1.4.3.4) enzymes MAO A and MAO B are FAD-containing proteins located on the outer face of the mitochondrial inner membrane, retained there by hydrophobic interactions and a transmembrane helix. The redox co-factor (FAD) is covalently attached to a cysteine and buried deep inside the protein [1]. Discovered as tyramine oxidase [2], an enzyme that metabolized pharmacologically active amines such as dopamine [3], MAO binds a range of small molecules, whose kinetic parameters are shown in Table 1.

MAO became an important drug target when a tuberculosis drug was found to improve the mood of patients. The large antidepressant market fueled the discovery of other irreversible inhibitors of MAO [4–6] that act by covalent attachment [7–9]. The common drugs such as selegiline (Table 1, used in the treatment of Parkinson's disease) modify the flavin, but binding to an amino acid residue was also reported for some compounds [10,11]. The major side-effect of treatment with the irreversible inhibitors is the tyramine-induced pressor "cheese effect" due to the inhibition of intestinal MAO A that normally metabolizes dietary tyramine found at high levels in aged cheeses [12]. The treatment of depression then switched to re-uptake inhibitors and drug discovery for MAO inhibition switched to reversible inhibitors, although the irreversible inactivators of MAO still remain important for treatment of certain types of depression. Furthermore, the increasing prevalence of neurodegenerative diseases has renewed interest in MAO inhibitors (MAOI) that can conserve the decreased levels of monoamine neurotransmitters in a deteriorating brain.

Although it is almost 100 years after the identification of monoamine oxidase, 70 years after its adoption as a drug target and 20 years after its structure was solved, some interesting questions still remain to be explored. These are outlined in this article as a starting point for new investigations.

Table 1. Some substrates, inhibitors and inactivators of MAO with their kinetic parameters.

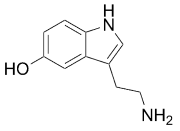
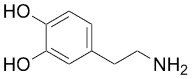
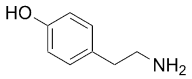
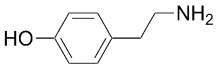
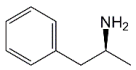
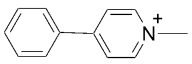
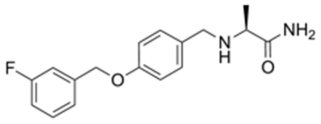
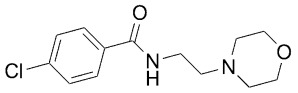
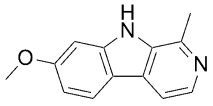
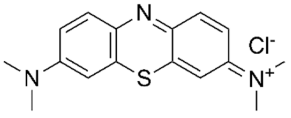
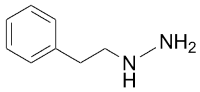
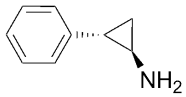
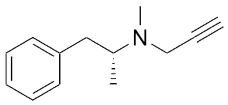
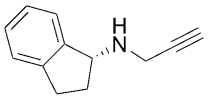
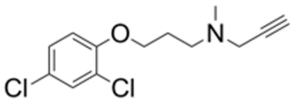
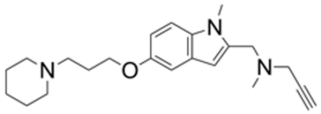
MAO A		Compound		MAO B	
K_M (μM)	V_{max} (pmol.min^{-1}.mg protein^{-1})	**Substrates** [A]		K_M (μM)	V_{max} (pmol.min^{-1}.mg protein^{-1})
137	228	Serotonin		1093	6.6
212	680	Dopamine		229	702
140	20	2-Phenylethylamine		4	309
127	182	Tyramine		107	343
K_i (μM)		**Reversible Inhibitors** [B]		K_i (μM)	
18		D-Amphetamine		250	
3		MPP^+ [c]		230	
365		Safinamide		0.45	
11.5		Moclobemide		>100	
0.005		Harmine		121	
0.028		Methylene Blue		5.5	

Table 1. *Cont.*

MAO A		Compound		MAO B	
K_I (μM)	k_{inact} (min^{-1})	**Irreversible Inhibitors** [C]		K_I (μM)	k_{inact} (min^{-1})
3.1	0.12	Phenelzine		50 [a]	0.9 [a]
7.7	0.78	Tranylcypromine		0.7	0.16
193	0.25	Selegiline [d]		0.1	0.53
9.7	0.007	Rasagiline [b]		0.7	0.05
0.03	1.8	Clorgyline		1.8	0.02
3.0	0.39	Contilisant [e]		4.6	0.19

Data: [A] from [13]; [B] summarized in [14] from references therein; [C] from [15] except [a] [16] and [b] [17]. [c] MPP^+ is 1-methyl-4-phenylpyridinium; [d] Selegiline is also known as L-deprenyl; [e] Contilisant is a multi-target compound which also inhibits cholinesterase and binds to the histamine H3 receptor [18].

2. What Is the Chemical Mechanism of Amine Oxidation? Is It the Same for Both MAO A and MAO B?

It is clear that MAO A and MAO B have different substrate and inhibitor selectivities (for example, [13,19–21]) defined by the distinct hydrophobicity and binding site architectures, but the key region close to the FAD is the same [21]. The chemical mechanism was substantially explored by traditional chemical approaches in the 1990s, but only four mechanisms for oxidation of the amine by MAO have attracted major attention: single electron transfer (SET), the polar nucleophilic mechanism or (as found in some other FAD-dependent oxidases) hydride extraction, and a derivative thereof, two-step hydride transfer [22] (see Figure 1). The strong chemical evidence for the single electron transfer mechanism came from in depth studies on the inactivation of MAO by cyclopropylamines and remains a probable mechanism for conversion of a cyclopropylamine into an activated product capable of forming a covalent bond either to the FAD or to a cysteine [10,23]. However, inability to detect a flavin semiquinone radical by electron paramagnetic resonance (EPR) during substrate turnover cast doubt on SET as the general catalytic pathway. Quantum chemical calculations for a SET mechanism for para-substituted benzylamines gave inverse correlation with experimental data, confirming that this is not the mechanism of substrate oxidation [24].

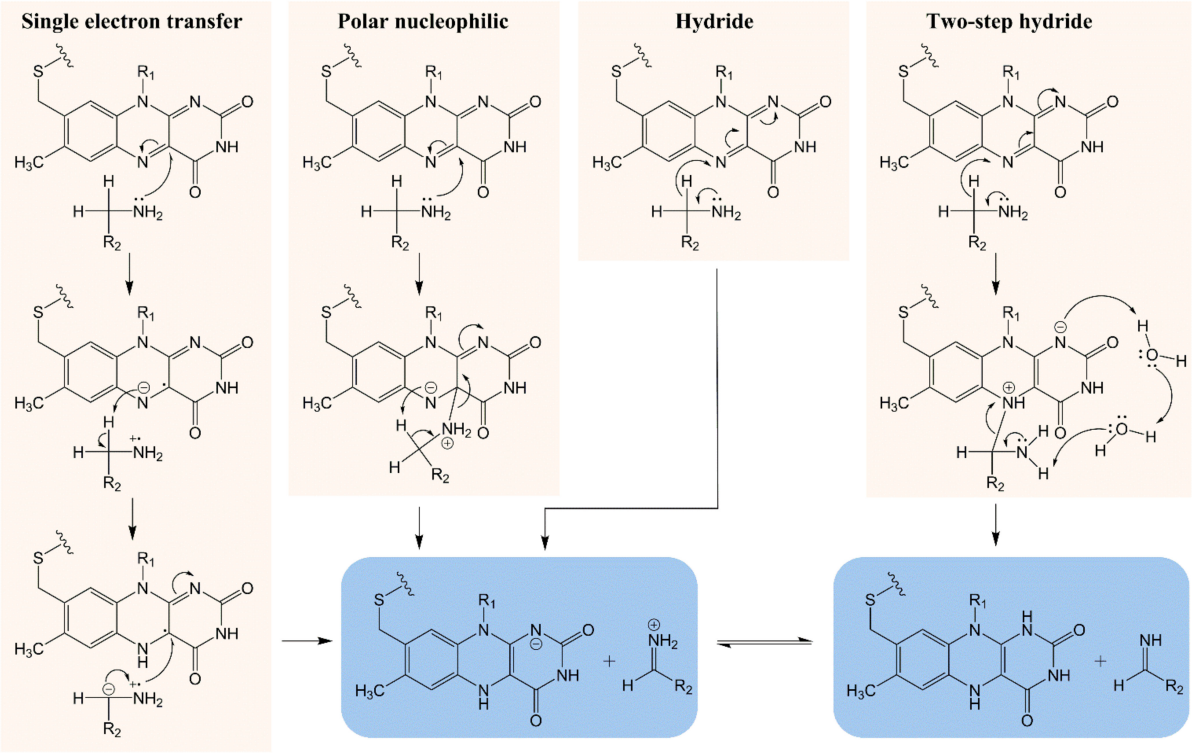


Figure 1. Chemical mechanisms proposed for MAO-catalyzed oxidation of amines.

In depth studies based on Hammett correlations were carried out in support of a polar nucleophilic mechanism for MAO A where a convincing positive correlation of oxidation rate with electronic effects of benzylamine substituents was obtained ($\varrho = 2$) [25]. In this study with MAO A, the more electron withdrawing character of the substituent, the faster was the rate of oxidation, consistent with the polar nucleophilic mechanism whereby a transient C4a adduct was formed with the flavin. In contrast, for MAO B, the substituents that more efficiently stabilize the developing electron density in the transition state slowed the rate, prompting the suggestion that MAO A and MAO B followed different mechanisms [26]. However, although the slower rate of MAO B with electron withdrawing substituents could be consistent with the hydride mechanism, nitrogen kinetic isotope effects for the oxidation of benzylamine by MAO B showed that the rehybridization of the N(5) nitrogen atom required for reduction of the flavin and the cleavage of the CH bond for oxidation of the amine were not concerted [27]. A quantum mechanics/molecular mechanics (QM/MM) study of benzylamine oxidation by the MAO also suggested that the transfer of electrons and proton was asynchronous, supporting the polar nucleophilic mechanism [28].

Hydride transfer was established for D-amino acid oxidase after high-resolution structures with bound substrates were solved giving insight into the probable transition state [29]. On the other hand, X-ray structures of MAO are available only with inhibitors bound (see, for example, [30]), but since computational modeling based on these structures became practical, evidence for a hydride transfer reaction in both MAO A and MAO B has been mounting [31–34]. In a QM/MM simulation of the rate-limiting step for the serotonin oxidation via hydride transfer to the N5 of the FAD in MAO A, the calculated barrier was 14.8 ± 0.8 kcal mol^{-1}, in good agreement with the experimental value of 16.0 kcal mol^{-1}. The two-step hydride transfer mechanism shown for MAO B proposes a hydride abstraction from the substrate and relaxation of the resulting flavin-substrate adduct into a neutral amine and fully reduced flavin. The second part of the reaction is presumably facilitated by two water molecules present at the binding site [34]. The activation free energy for the hydride transfer in this computational study was shown to

be roughly two-times smaller (24 kcal mol^{-1}) than for any other oxidation mechanism by MAO B.

Whether further experimental or modeling work would give clearer evidence for the same hydride mechanism in both MAO A and MAO B is not certain. However, could two enzymes with such similar catalytic sites support non-identical chemical mechanisms? Using Hammett correlations as in the example above has proved invaluable in many chemical mechanistic studies. However, inductive and resonance effects of the substituents on the aryl rings of reactants/substrates are rarely the only (although might be the prevailing) factors governing the reaction kinetics. It has been demonstrated for MAO, for instance, that steric hindrance in 2′-aryl substituted analogs of MPTP had a significant effect on the determined Hammett values [35], and not only intramolecular but also intermolecular steric effects and hydrophobic/hydrophilic interactions between the aryl substituents of a reactant and its surrounding can be a source of deviation from linear Hammett plots (e.g., [36–39]). These contributions to non-linearity are especially relevant when studying mechanisms of enzymatic reactions since these take place within a tight binding site where the degree of interactions of a particular substituent with amino acid residues of the immediate surrounding can be considerable and distinct from that in the bulk solvent. In the most severe cases, the rate-determining step or even the reaction mechanism itself can change with the substituent [40,41]. Therefore, could the differing kinetic data (that provide indirect insight into the catalytic mechanism of MAO) mostly reflect the steric and/or other intermolecular interactions imparted by a distinct catalytic site (Figure 2) which tilts the apparent activity of MAO A or MAO B to one or the other kinetic pattern in an otherwise identical catalytic mechanism?

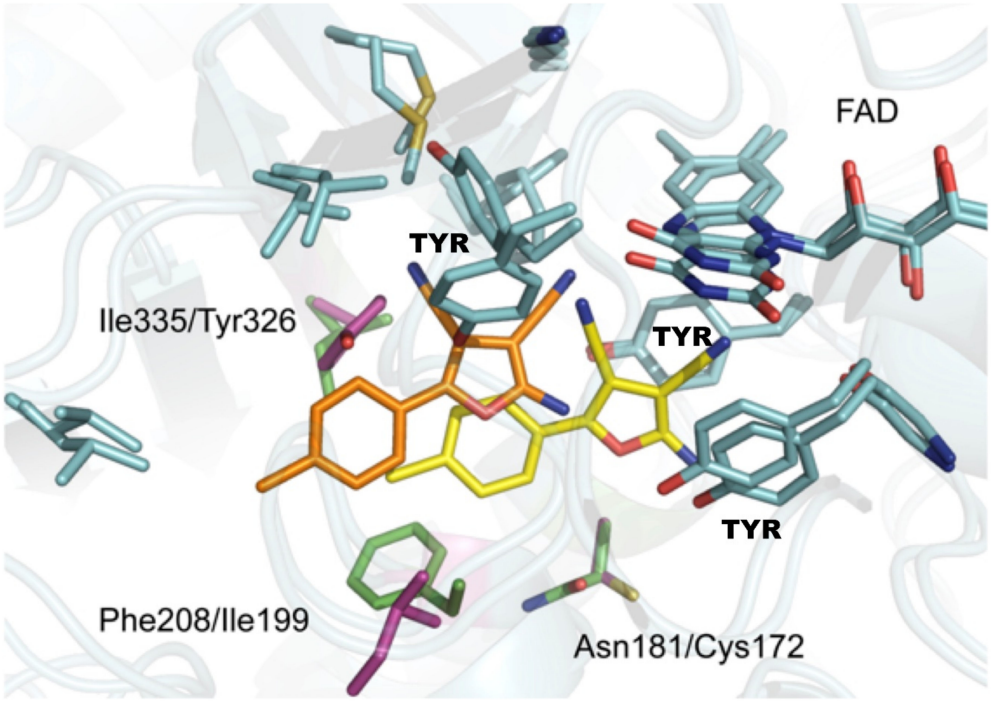


Figure 2. Small molecule binding in the active site of MAO A and MAO B. Representative binding modes for the inhibitor 2-amino-5-(4-chlorophenyl)furan-3,4-dicarbonitrile bound to MAO A and MAO B obtained from molecular dynamics simulations [42]. The inhibitor is shown as orange sticks in MAO A and yellow sticks MAO B. Residues common to MAO A and MAO B are shown as blue sticks, with the key "aromatic cage" tyrosines labelled in black (**TYR**). Residues that differ are shown in green for MAO A and in magenta for MAO B and identified by the MAO A residue number followed by the MAO B residue number. These residues have been reported to be important in selectivity for small molecule binding. The flavin cofactor with the bent isoalloxazine ring is shown as blue sticks for both MAO A and B.

3. The Oxidative Half-Reaction: How Does Oxygen Access the Reduced Flavin, and Is It Blocked or Facilitated by Ligands?

The regeneration of oxidized MAO, which completes MAO catalytic cycle, has been less intensely studied. At a minimum, more experiments are needed to explain the very different K_M values for oxygen (MAO A at 0.06 mM but MAO B at 0.28 mM [43]). The kinetics of oxidation of the amine substrate and the accompanying reduction of the FAD in MAO have been studied in detail [44–48], but very little is known about how oxygen accesses the reduced flavin to restore the oxidized FAD and produce hydrogen peroxide (H_2O_2), the product of the second half-reaction and a potential cause of degenerative effects [49].

In common with other oxidases, MAO has the FAD:N(5)-water-Lys (305 in MAO A) motif thought to be key for oxygen activation [50]. By positioning oxygen in high affinity sites, the likelihood of O_2 reacting with the correct stereochemistry with FAD is increased and generation of undesirable reactive oxygen species is minimized. The reaction takes place between oxygen and the C4a position of the flavin. Key factors influencing the reaction include accessibility, the distribution of charged and polar groups to favor electron transfer between the two reactants, and the extent of stabilization of the C4a-hydroperoxyflavin intermediate [51,52].

How does the oxygen get to the deeply buried site? In other oxidases, multiple pathways have been identified [51]. Oxygen diffusion to the reduced co-factor is most likely via tunnels that allow regulation of oxygen access [51]. Studies on MAO similar to those that revealed multiple pathways in cholesterol oxidases [53] would be useful. Could similar pathways in MAO provide insight into the substrate acceleration of re-oxidation (see below)? Moreover, induced fit with substrate alters the O_2 locations and opens alternative channels [54]. Where are the oxygen access channels in MAO and does substrate or product binding to reduced MAO change access or activation?

4. Kinetics: Can Simulation Explain the Alternative Pathways in Turnover; Why Substrate Accelerates Oxidation and the Influence of Two Forms for Binding Inhibitors?

4.1. Both the Oxidized and Reduced Forms of MAO Can Bind Ligands during Turnover

Early work varying both benzylamine and oxygen indicated a ping-pong mechanism for rat MAO [55,56], but later kinetic studies demonstrated that some substrates followed a ternary complex mechanism (Figure 3) [45,57,58]. Spontaneous hydrolysis of the imine product to the aldehyde plus ammonia, at least some of which may occur in the active site, complicates the picture [59]. However, hydrolysis of imine in the active site is probably not the prevailing mechanism, but rather, it is primarily non-enzymatically hydrolyzed outside the enzyme [60]. The number (and orientation) of water molecules in the active site depends on the particular isozyme (MAO A versus MAO B) and the nature of the substrate [49,61,62]. Many water molecules can be of structural type (e.g., involved in hydrogen bonding), making them very unlikely to take part in any type of reaction [63]. Nonetheless, imine hydrolysis in the active site can still be considered plausible. Aldehydes are very poor uncompetitive inhibitors [60] so can be considered to dissociate easily, but does ammonia dissociate more slowly? With a pK_a of ammonia being 9.2, it is not difficult to imagine a proton abstraction by ammonia from an acidic amino acid residue in the active site, resulting in a protonated species which can be effectively "trapped" by favorable cation-π interactions with the tyrosine residues that constitute the "aromatic cage" [63,64]. From results using a benzylamine analog oxidized to a spectrally distinct imine product, it has been argued that the reduced enzyme-imine complex is reoxidized before the bound imine signal is lost [59]. If either ammonia or imine remained bound, free reduced enzyme would be minimal, and no new ligand could bind. However, the kinetics demonstrating binding of inhibitor to reduced enzyme indicate that free reduced enzyme is present during turnover [57,58,65].

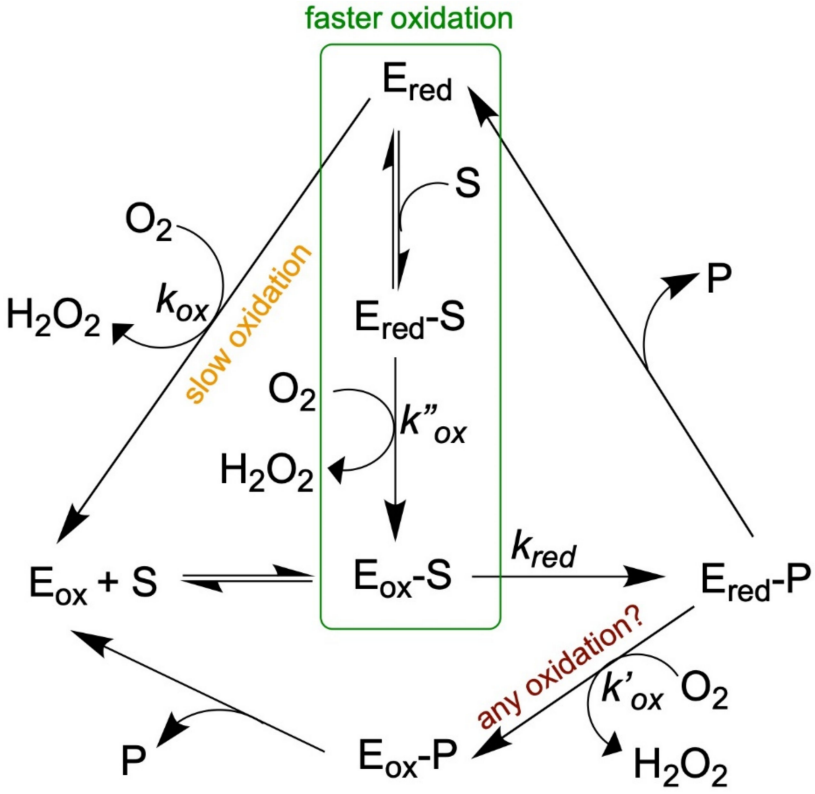


Figure 3. Alternative pathways for oxidation complicate MAO kinetic studies.

Ultimately, whether a pool of free reduced enzyme builds up during turnover will depend on the rate constants for the half-reactions, the concentrations of the substrates (amine and oxygen), and the affinities of the oxidized (E_o) and reduced (E_r) forms of the enzyme for their ligands (see Figure 3). Although reduction of the FAD by the amine substrate is the slowest step in the reaction catalyzed by MAO, there are three possible pathways for the reoxidation of the reduced FAD co-factor. The rate of reoxidation of the reduced MAO B without ligand is slow (k_{ox}, 1.8 s^{-1} at 0.28 mM oxygen) and whether E_{red}-P is oxidized before dissociation of the imine product is questioned. When some substrates are preincubated with reduced MAO, the rate of oxidation increases 2–100 times [45]; so for those substrates, the pathway in the green box dominates. Based on data from [45], 2-phenylethylamine (PEA) reduces bovine MAO B at 572 s^{-1} (k_{red}), rapidly generating E_{red}, but the steady-state turnover rate is 3.6 s^{-1}. The imbalance in rates means that the MAO is mostly reduced (E_{red}) during steady-state oxidation of PEA. Based on steady-state and half-reaction studies, oxidation of PEA is mainly via the binary pathway. With benzylamine as substrate, the reduction and reoxidation rates are similar (10.9 and 7.6 s^{-1}, respectively), and reoxidation is mainly via the ternary complex pathway indicated by the green rectangle. It should be noted that reoxidation rates are influenced by the concentration of oxygen ($[O_2]$). Even with these similar rates of reduction and oxidation, some intermediate E_{red} is available for binding of inhibitor as has been demonstrated in bovine MAO B [57] and for human MAO B [65]. That the affinities of the oxidized (E_o) and reduced (E_r) forms of the enzyme for inhibitors can differ was demonstrated for the tricyclic antidepressant amitriptyline which gives a K*i* of 0.5 mM with E_o but 2.5 mM with E_r [66]. The question is important for the drug design process because IC_{50} measurements used to screen new compounds will vary with the proportion of reduced enzyme present during turnover which depends upon the assay and substrate used [65,66].

The occurrence of differential binding to E_o and E_r was established by in-depth kinetic investigations in vitro [65]. In laboratory assays, the O_2 concentration in buffer at 30 °C (0.22 mM) is below the K_M for O_2 with MAO B (0.28 mM) [43], and the oxygen content drops with temperature. Although the kinetic isotope effects in air-saturated buffer (21.1%

or 101.3 kPa oxygen) of 5–10 show that amine oxidation is the slower step [46], the rate of reoxidation at sub-saturating oxygen conditions will influence turnover more as the oxygen concentration decreases below its K_M, especially at low substrate (amine) levels. (See more information on the kinetics of oxidation below.) In cell culture at 37 °C and 21% oxygen, a dissolved extracellular concentration of 0.21 mM was measured, but depending on conditions, oxygen concentration intracellularly was only 0.175 mM [67]. However, this is now considered hyperoxia rather than physiological normoxia. Human tissues have oxygen concentrations varying around 6%, but inside the cell, it is 1.3–2.5% [67], one-tenth of that for in vitro experimental concentrations. Could simulations explore MAO kinetics and redox status (proportions of E_o and E_r) in the brain?

4.2. Substrate Stimulation of Reoxidation-Thermodynamic or Kinetic?

That the slowest step in the MAO reaction is the reductive half-reaction (oxidation of substrate amine to imine) is clear from studies involving deuterated substrates where large kinetic isotope effects of 5–14 were obtained [25,46,48,68–71]. However, the oxidative half-reaction of MAO measured in isolation is slower than the observed steady-state turnover rate for many substrates [45]. By pre-incubating chemically reduced MAO with substrate before rapidly mixing it with oxygen, it was demonstrated that the substrate stimulates the rate of reoxidation, whereas, for MAO A, a product (MPP^+) or an inhibitor (D-amphetamine) inhibit the rate. Moreover, different substrates give different increases in the slow rate of reoxidation of free reduced enzyme (1 s^{-1}) found with both MAO A and MAO B. For MAO A with kynuramine, the rate of flavin re-oxidation was 120 s^{-1}, with benzylamine 23 s^{-1} and with MPTP 40 s^{-1}. For MAO B, the rate of re-oxidation of the flavin in enzyme saturated with kynuramine was 2.2 s^{-1}, benzylamine 7.6 s^{-1}, and MPTP 6.0 s^{-1} [45]. Therefore, is it the imine or ammonia still bound that facilitates the oxidation (although MPP^+ clearly inhibits) or does the binding of new substrate to the reduced enzyme govern the kinetics of oxidative half-reaction? Inhibition kinetics [57] do show that free enzyme is available before re-oxidation of the reduced flavin, so perhaps substrate acceleration is the dominant pathway for the normal substrates, but how is the effect achieved?

Measurement of the redox potential of the reductive half-reaction for MAO A in the absence and presence of substrate suggested that substrate increased the redox potential [72] but similar experiments in MAO B did not [73]. A change in redox potential would indicate a change in the conformation or environment of the FAD, but it is more likely that the reported MAO A change was due to lack of equilibrium between the dye and FAD. However, the environment of the flavin has long been known to influence the redox potential [74], and substrate (or inhibitor) binding is likely to alter that environment. Indeed, the shifts in the circular dichroism spectrum indicated differences in the FAD region when substrate was added [75]. The differences between the oxidized and reduced circular dichroism spectra also indicate a change in the FAD environment or shape (see below).

5. Is There Potential for Applied Use of MAO?

MAO turnover is slow (3–10 s^{-1} [45]) but specific (the *pro-R* hydrogen from the α carbon next to the amine is removed [76]). Tighter substrate cavity of MAO B results in increased steric hindrance and in distinctive amino acid residue interactions with small molecules. This makes MAO B more sensitive to the absolute configuration at chiral centers of enantiomeric substrates and inhibitors [20] with potential for high value chemical synthesis.

Specificity is also governed by hydrophobic and hydrophilic regions of the cavity. The environments of both substrate cavities are generally hydrophobic, but MAO B is slightly more so. Consequently, by transforming some regular MAO B inhibitors into quaternary ammonium salts, selective MAO A inhibitors were obtained [77]. Within each cavity, docking of variants within the many classes of inhibitors has demonstrated the importance of specific interactions such as hydrogen bonding. Specially, MAO A

Phe208-Ile335 and MAO B Ile199-Tyr326 pairs (see Figure 2) were suggested to be major determinants which govern the differential inhibitor specificities of the two isozymes [78]. Site-directed mutagenesis studies showed that Ile335 in MAO A and Tyr326 in MAO B are key amino acid residues determining substrate and inhibitor specificities in human and rat MAO A and B [79–81]. By tempering with these two residues, the spatial and chemical architecture of the substrate cavity is altered, and MAO B selectivity starts to mimic that of MAO A and vice versa. A change in selectivity was also induced by the single mutation Phe208 to Ile199 [82]. However, the wide range of effective inhibitors demonstrates that affinity for a ligand is determined by multiple interactions within the large cavity (for examples, see recent reviews: [83–85]).

Purified MAO is not stable enough for most applications, but MAO electrodes incorporating membrane-bound MAO have been devised for measurement of amines, for example, for serotonin using MAO A [86,87]. For electrode use, an alternative to oxygen as the electron acceptor in the oxidative half-reaction is desirable. Methylene Blue and similar dyes are known acceptors [88,89], but performance could be improved by rational engineering of the oxygen pathway or reaction site [90]. This has been demonstrated for MAO A by mutating Lys305: after reduction by serotonin, the K305M MAO A reoxidation by oxygen was much slower but electrons could pass instead to a quinone [91].

In contrast to the mitochondrial MAO, the *Aspergillus niger* enzyme, MAO N is more tractable for protein engineering and large-scale production although the FAD is not covalently bound to the enzyme [92–94]. MAO N is suitable for chiral catalysis and use of whole cells avoids the limitation of FAD supply [95,96]. Whole cell methodologies might be the best way forward for any use of mitochondrial MAO, but tuning MAO N to the job seems the better option.

Perhaps also of interest for applications in biological tissues or in vivo is the observation that geometric isomers of *cis*- and *trans*-1-propargyl-4-styrylpiperidine distinguish between MAO A and MAO B. The *cis* isomers are potent human MAO A irreversible inhibitors whereas the *trans* analogues inactivate only MAO B both in vitro and in mouse brain [97].

Overall, drug design remains the major applied opportunity for treatment of depression, neurodegeneration or cancer [98]. Identification of key interactions at the catalytic site fuels innovation towards new lines of MAO inhibiting drugs with enhanced selectivity and potency.

6. Importance of Flexibility

6.1. Is Flexibility Important in the Reaction?

Crystal structures present a frozen view of enzymes, but from this accurate foundation, rapidly advancing computational approaches reveal a much more dynamic nature of enzymes in their physiologic environment, including, for MAO, at the interface of the cytosol and the mitochondrial outer membrane. Experimentally, MAO flexibility is seen as changes in the circular dichroism spectra of both flavin and aromatic amino acid environments after ligand binding or redox change [75]. An early modeling test (on the MAO B structure from which the reversible inhibitor, isatin, had been removed) fixing the amino acids around the flavin indicated no hindrance to flattening of isoalloxazine ring from the 30° bend seen in the crystal structure of the enzyme-bound flavin [99]. The isoalloxazine ring in many flavoproteins changes from flat to a bent ring upon reduction, but all crystal structures of MAO whether oxidized (reversible inhibitor bound) or reduced (irreversible inhibitor attached to the FAD) show a 30° bend along the N(5)-N(10) axis [78]. It is hypothesized that this strain at the coenzyme binding site may have catalytic relevance, mainly increasing the nucleophilicity of N(5) and electrophilicity of C(4a) [100]. Any movement of FAD or protein residues could also alter the oxygen accessibility, crucial for MAO turnover. If structural perturbations could be used effectively to steer the electronic characteristics of the cofactor and thus regulate the catalytic activity, why does flavin in MAO A and MAO B adopt a bent position in crystals regardless of its redox state?

Turning to the flexibility of the protein, induced fit within the active site is evidenced by the slow onset of full inhibition by larger reversible inhibitors (even 1-methyl-4-phenylpyridinium (MPP^+) for which perturbation of the flavin spectrum showed an on-rate of 10^8). Slow on (30 min) and off (6 washes) rates were also observed in reversibility experiments with safinamide [101]. Molecular dynamics now confirm considerable movement in active site in addition to alternate binding modes obtained by docking. Enzyme tertiary structure adjustments of the active site environment are also necessary to accommodate the irreversibly bound inhibitors, which cannot adopt the most stable structural conformation otherwise [102]. Therefore, what constitutes the line between MAO flexibility and rigidity allowing for a number of structurally distinct (but related) substrates to be oxidized by the enzyme while assuring that certain biological functions are properly regulated on account of substrate specificity?

Flexibility (and rigidity) could also be important for crosstalk between the monomers in the MAO dimer. Pulsed electron paramagnetic studies demonstrated that both MAOs from human and rat are dimeric in the membrane [103]. Does dimer crosstalk play a role in kinetics or in ligand binding? Although there was some speculation in older literature about occupancy of one site changing the activity of the other, this topic needs evidence.

6.2. The Influence of the Membrane on MAO Activity

Simple steady-state kinetic experiments showed that membrane-bound MAO gave a lower K_M for some substrates compared to purified enzyme. For example, MAO A had a 10-fold lower reversible K*i* for clorgyline [104]. In experiments studying solvent accessibility to the active site using spin-labeled derivatives of the inactivator, pargyline, no difference between detergent-extracted and membrane-bound MAO was found [105]. However, lower K*i* values for the initial reversible inhibition of MAO by propargylamine inactivators in membrane preparations was demonstrated in kinetic studies. The close association of MAO with the membrane and the close proximity of the entrance to the active site to the membrane surface might be expected to influence the dynamics of substrate access or product exit due to steric hindrances and the flexibility of the substrate entrance loop (residues 99–112) [106,107]. When this question was examined using molecular dynamic simulations, effects were indeed found. The membrane influenced the fluctuations of two loops at the entrance to the active site cavity (residues 85–110 and 155–165 in MAO B) [108]. Membrane control of the active site access was reinforced by the differences in the temperature-dependence of benzylamine oxidation by MAO B between purified enzyme and the same enzyme attached to nanodiscs [109]. (However, it should be noted that the preparation used for these experiments had a completely different spectrum from MAO B purified in at least three other laboratories.) Exploring the influence of the membrane using long molecular dynamic simulations, the same authors looked for oxygen tunnels leading to the FAD and found two entrances, one on each side of the gating loop (residues 99–112). Residues at both entrances are closely associated with the membrane. Given the insight from these computational studies, new investigation of previously observed lipid effects on rat mitochondria MAO could provide further understanding of the importance in vivo [110–114]. Could alterations in the membrane composition (from diet, for example) have consequences for activity, raising or lowering the monoamine pool in the brain?

6.3. Does the Oxidation of MAO Thiols Have Any Importance In Vivo?

Continuing the theme of biological influence from chemical changes, do the solvent accessible cysteines influence activity? Both MAO A and MAO B form a covalent 8α-thioether linkage with the flavin (at Cys407 and Cys398, respectively) and contain 8 other cysteine residues in reduced (SH) form. Although 7 of the 9 cysteines are in conserved sequence locations, only MAO B has one that interacts with ligand in the active site (Cys172) [115,116]. Free thiol groups in proteins are subject to various modifications from reaction with reactive species generated by mitochondria [117,118] and thiol modification can both regulate and damage cell function [119]. Although located on the cytosolic

surface of the mitochondrial outer membrane, the 8 modifiable cysteines in MAO [120–122] are close to the greatest cellular source of reactive oxygen species, the electron transport chain on the inner membrane. The initial study where each Cys was mutated to serine demonstrated individual effects of each on MAO activity. Most of the mutants behaved like the wild-types, but complete loss of activity was observed for MAO A Ser374 and MAO B Ser156 and Ser365 mutants [123]. Later, Cys365 was labeled specifically during inactivation of MAO B with *N*-cyclopropyl-*N*-α-methylbenzylamine, indicating its involvement in the inhibitory pathway [124]. The equivalent Cys374 in MAO A substituted with alanine gave k_{cat}/K_M values 30% lower than for the wild type [122]. Chemical modification of the thiols revealed different susceptibility: MAO A was more rapidly inactivated by *N*-ethylmaleimide (half-life about 3 min) than MAO B (half-life about 8 h) [121]. Moreover, Cys266 and Cys5 seem to be the only two thiols modified in clorgyline-inactivated MAO A and pargyline-inactivated MAO B, respectively, which demonstrates that inhibitors, as well as substrates, stabilize the enzyme structure and increase the rigidity [116]. Does occupancy of the active site by the amine substrate prevent oxidation of cysteine residues in mitochondrial MAO and to what extent?

The presence of reactive oxygen species around mitochondria in the cell can oxidize protein thiols in the vicinity, so thiol modification could be significant for catalysis under physiological conditions. Further development and application of chemical methods to detect, distinguish, and time modify cysteines in the cell or tissues would allow better understanding of any consequences from modifications of cysteines in MAO [119,125,126].

7. Biological Questions

7.1. Does Variation in the Calcium Ion Concentration Change MAO Activity?

In neurons, ion fluxes during the action potential result in transient changes in the calcium ion concentration in the cytosol, but uptake by the endoplasmic reticulum and mitochondria quickly restore the normal low micromolar level. Calcium (Ca^{2+}) was found to increase MAO activity in mitochondria and cells [127–130]. When a short burst of Ca^{2+} was induced in mice by a calcium channel agonist, the increase in MAO A activity was transient [130]. In vitro, preincubation of membrane-bound hMAO with 1 mM Ca^{2+} increased the MAO A maximum velocity 2-fold but did not change MAO B [131]. No other divalent cation increased activity but zinc ion was strongly inhibitory. More physiologically relevant, a 20% increase in activity was observed when Ca^{2+} was increased from 10 nM to 10 uM, the normal range for calcium signaling. How does Ca^{2+} exert this influence on activity? Is the ion size the determining factor? Why is only MAO A activated? A calcium-sensitive increase in MAO A activity increased the production of peroxyradicals in hippocampal cultures, and this was postulated to have implications for neuronal damage in Alzheimer disease [132]. Is this calcium-sensitive stimulation of MAO A activity increase relevant in the cell and in pathology?

7.2. Does MAO A Moonliaght in the Cell?

MAO metabolizes neurotransmitter amines in nervous tissue and biogenic amines in all cells so that alterations in activity alter signaling amine levels. Sustained changes in monoamine levels trigger adaptations in levels of other proteins, including receptors. For MAO A itself, putative non-catalytic roles for the protein have been proposed, involving either direct protein-protein interaction or a signaling relay influencing gene expression. The association of some Alzheimer's disease (AD)-related presenilin-1 variants with depression led to studies that demonstrated a direct interaction between presenilin-1 and MAO A proteins and a change in MAO A activity [133,134]. MAO A knockout mice revealed the importance of serotonin and MAO A in apoptosis during brain development [135]. MAO A levels are influenced by both genetic and environmental factors, and MAO A activity influences psychopathologies [136,137]. The anorexigenic hormone leptin that protects mitochondrial function regulates the expression and activity of MAO B [138]. Leptin also reduced the increase in MAO A and MAO B expression found in hippocampal neurons

treated with the Alzheimer's disease peptide Aβ. In Alzheimer's disease, which leads to cell loss in the brain, an increase in MAO B was evident but MAO A also increased in some regions. Intriguingly, co-regulation of MAO A and MAO B levels observed in normal post-mortem brains was lost in the hippocampus but not in the cortex of Alzheimer brains [139].

When MAO A activity in human neuroblastoma cells was knocked down to about 30% that of control cells, ATP was increased, reactive oxygen species were decreased, and the cells were protected against apoptosis. The effects were linked to electron transport because although inhibition of electron transport up-regulated MAO A in control cells, the low MAO A activity in the knock-down cells protected mitochondria from inhibition [140].

Although a genomic study found MAO A to be down-regulated in some cancers [141], it is clearly elevated in aggressive prostate cancer and classical Hodgkin's lymphoma [142,143]. A small Phase 2 clinical trial found that the irreversible MAO inhibitor, phenelzine, successfully decreased markers of prostate cancer, opening a new application for MAO A inhibitors [144].

Keeping in mind the location of MAO at the interface between the cytosol and mitochondria, untangling the many interactions for MAO and the network of signaling and gene expression will require considerably more cellular and genetic research.

7.3. Species Differences and Mutations?

Kinetic studies identified differences in inhibitor binding to MAOs from different species [145–149]. Although mammals all have two forms of MAO, fish have only one [150]. After the structures of MAOs were published, a detailed examination of the kinetics and structures of MAOs from rat and human provided better understanding of the differences [105,151]. With different types of cells used in many pharmacological and toxicology studies, researchers should be aware also of differential expression of MAO A or B in each cell type [152]. The question in this section is curiosity-driven: species differences are seen in the active sites of MAO A and MAO B, but human mutations altering active site amino acid residues to influence activity or ligand binding have not been identified from clinical studies. Known human variants identified by genetic studies are sequence repeats that alter expression levels with behavioral consequences [153–157].

One theoretical study has provided the basis for a lower activity based on a mutation in the middle of the MAO B active site. When tyrosine 326 in MAO B is changed to isoleucine (as found in MAO A-so the mutant oxidizes serotonin better), the activity (k_{cat}) with phenylethylamine dropped to 27% of the wild-type, and the K_M increased [79]. When the oxidation of the amine was simulated, reaction free energy profiles showed that the barrier in the mutant was 1.06 kcal mol^{-1} higher, due to loss of electrostatic interactions and dielectric shielding [158]. Are there similar variants in the human populations and could new experiments and clinical studies demonstrate consequences of the lower activity giving higher amine levels in the brain for human behavior?

7.4. The Pharma Debate-Reversible or Irreversible Inhibition?

The effect of a successful drug is governed by binding affinity and by pharmacokinetics. For a high affinity reversible inhibitor, side effects should be low, and the inhibition is easily reversed. Indeed, it might be desirable that a surge in the amine substrate concentration would out-compete the inhibitor. For irreversible inhibitors, reaching a steady-state level of inhibition might take longer if the dose is low, but the effect would persist until the inactivated enzyme was replaced by a new protein molecule.

To achieve the desired clinical effect in depression or Parkinson's disease, MAO inhibition must be greater than 75% [159]. Despite the rapid inhibition of MAO that would occur in vitro (immediate for reversible inhibitors and minutes for irreversible inhibitors) and the detection of MAO using ^{11}C-deprenyl in positron emission tomography (PET) scans recorded from 25 min after the infusion [160], clinical improvement is slow [161]. At the end of treatment, a "wash-out" period of three weeks is recommended for the

irreversible inhibitors to allow the synthesis of new protein, so activity resumes at the slow pace of turnover-the half-life of MAO B in baboons was measured by PET using labeled L-deprenyl as 30 days [162]. For reversible inhibitors, clearance of the drug from circulation should result in rapid return of MAO activity, but a minimum of two weeks washout is still recommended.

The question of whether a reversible MAOI or an irreversible MAOI provides better treatment has mostly been investigated for Parkinson's disease for which the irreversible selegiline (or the similar drug, rasagiline) and the reversible safinamide are commonly used to delay the need for levodopa (L-dopa) treatment. Selegiline has neuroprotective effects [163] and antidepressant action, but tyramine potentiation fears and dietary restrictions persist, except for the transdermal form. Safinamide has other effects beneficial against PD including blockage of voltage-dependent sodium channels and has low tyramine potentiation [164]. When reversible and irreversible inhibitors are compared, both have been found to be effective as adjuvant therapy to delay or reduce the need for L-dopa [165]. However, a meta-analysis comparing MAOI alone or with L-dopa showed that selegiline was the most effective drug and that both it and rasagiline were more effective than safinamide (selegiline > rasagiline > safinamide) [166].

Further study of clinical outcomes with irreversible versus reversible inhibitors in depression would also be useful. Reuptake inhibitors are first choice for depression, then reversible MAOI, while irreversible inhibitors are used when other treatments fail. This is partly due to the side effects that require dietary restrictions but probably more due to inexperience with these drugs [167,168]. Certainly for Parkinson's disease, prescribing habits vary from country to country [165].

Much recent effort has gone into designing reversible MAOI, especially for MAO B and Parkinson's disease. Many scaffolds are used, but particularly successful compounds have been found amongst coumarin derivatives, which are competitive inhibitors with inhibition constants as low as 0.25 nM [169–172]. However, what if irreversible inhibitors provide more stable inhibition of MAO, especially in contrast to drop in inhibition unless there is strict compliance with the dosing regimen for reversible drugs? Certainly, multi-target compounds designed for use in multi-factorial neurodegeneration such as Alzheimer's disease have employed the propargylamine moiety to give irreversible MAO inhibition leaving more flexibility to adapt the rest of the molecule to give high affinity binding to other targets such as the cholinesterases. For example, the propargylamine moiety for MAO inactivation has been combined with inhibition of the cholinesterases and antagonism at histamine H3 receptors in one molecule with suitable drug-like properties [18,173]. However, do these multi-target species really "outperform" a drug mixture composed of individual inhibitors in clinical use?

Therefore, what should drug research for MAO seek: reversible or irreversible? It is still a debate, but the original irreversible classes on MAO inhibitor drugs are still an essential part of clinical practice, with the reversible drugs appearing somewhat less effective.

8. Conclusions

After 100 years of research on the MAO enzymes and 60 years on the MAOI, there remain still interesting questions to explore for neurologists, pharmacologists, enzymologists, computational experts and chemists. Have fun!

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