Substrate-Specific Enhancement of the Oxidative Half-Reaction of Monoamine Oxidase†

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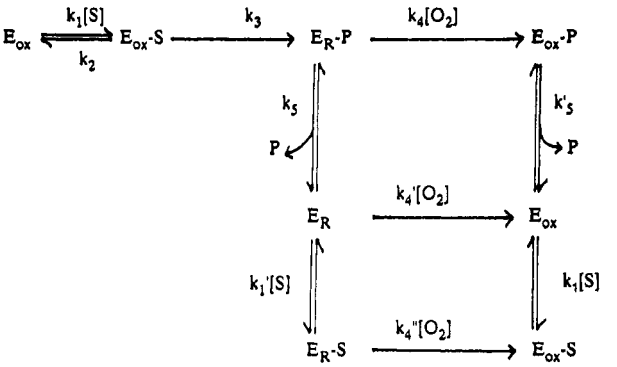
ABSTRACT: Monoamine oxidases A and B have identical flavin sites but different, although overlapping, amine substrate specificity. Reoxidation of ternary complexes containing substrate is much faster than of free enzyme, and the enhancement is greater in the A form than the B form. The oxidative half-reaction was studied with a variety of substrates to elucidate the specificity of the effect and to probe the different influences of substrate on the flavin reoxidation in the two forms of the enzyme. The second-order rate constant for the reoxidation was highest with monoamine oxidase A when kynuramine was the ligand (508×10^3 M^{-1} s^{-1}) compared to 4×10^3 M^{-1} s^{-1} in its absence. MPTP (166×10^3 M^{-1} s^{-1}) also enhanced reoxidation well, but indole substrates stimulated only poorly (e.g., tryptamine, 29×10^3 M^{-1} s^{-1}; serotonin, 50×10^3 M^{-1} s^{-1}). For the A form, the reduction of the flavin was rate-limiting in all cases. For the B form, reoxidation was rate-limiting for β-phenylethylamine and contributed to the determination of the overall rate with several substrates. The ratio of the enhanced rate of oxidation to the rate of reduction correlated with the redox state of the enzyme in turnover experiments. All the observations are consistent with alternate paths of reoxidation, via either free enzyme or a reduced enzyme–substrate complex. The flux through each path is determined by the relative dissociation constants and rate constants.

Mitochondrial monoamine oxidases catalyze the oxidative deamination of a variety of primary, secondary, and tertiary amines and are important in controlling the level of biogenic amines. The A and B forms of the enzyme have identical flavin sites (Nagy & Salach, 1981) but quite different although overlapping substrate specificities [for a review, see Singer (1991)]. Previous studies have shown that, for both the A and B forms, the reaction pathway can involve a ternary complex of reduced enzyme–substrate–O_2 and that reoxidation of the complex with substrate can be two orders of magnitude faster than that of the free enzyme (Ramsay, 1991; Ramsay et al., 1987). The mechanism is determined by competition between the alternate pathways (Scheme I) on the basis of the relative rate constants and dissociation constants.

The rate of the reoxidation of reduced MAO[1] with MPTP as the ligand was quite different from that with the common primary amine substrates, namely, kynuramine for MAO A or benzylamine for MAO B. A range of natural and other substrates have now been studied to show that each substrate stimulates the oxidative half-reaction differently depending on its structure. The steady-state parameters and those for the reductive and oxidative half-reactions were studied to seek the basis for these differences between the amine substrates.

The MAO A used in these experiments was the human liver form, which has been expressed in yeast (Weyler et al., 1990). The sequence of this human liver gene has been determined (Bach et al., 1988), but only a small part of the sequence of the placental enzyme (Chen & Weyler, 1988) used in previous work (Ramsay, 1991) is known. Steady-state kinetics revealed negligible differences between the liver and placental forms for the primary amines, but the tertiary amine, MPTP, and its derivatives were oxidized significantly faster by the liver enzyme (Tan et al., 1991). The basis for these differences is not yet known.

Scheme I: Alternate Pathways of Reoxidation of Reduced Monoamine Oxidases via Binary or Ternary Complexes



In this paper, we describe how substrates enhance the rate of reoxidation to different extents in the same enzyme and how in MAO A from two sources and in MAO B, all of which have the same sequence at the flavin site, the same substrate results in very different enhancement of the rate of reaction of the reduced enzyme with oxygen.

MATERIALS AND METHODS

Reagents. Benzylamine, kynuramine, β-phenylethylamine, tryptamine, 5-hydroxytryptamine (serotonin), 5-methoxytryptamine, and *n*-octyl β-D-glucopyranoside were purchased from Sigma Chemical Co. MPTP, $MPDP^+$, and MPP^+ were

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[1] Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium; MAO, monoamine oxidase.

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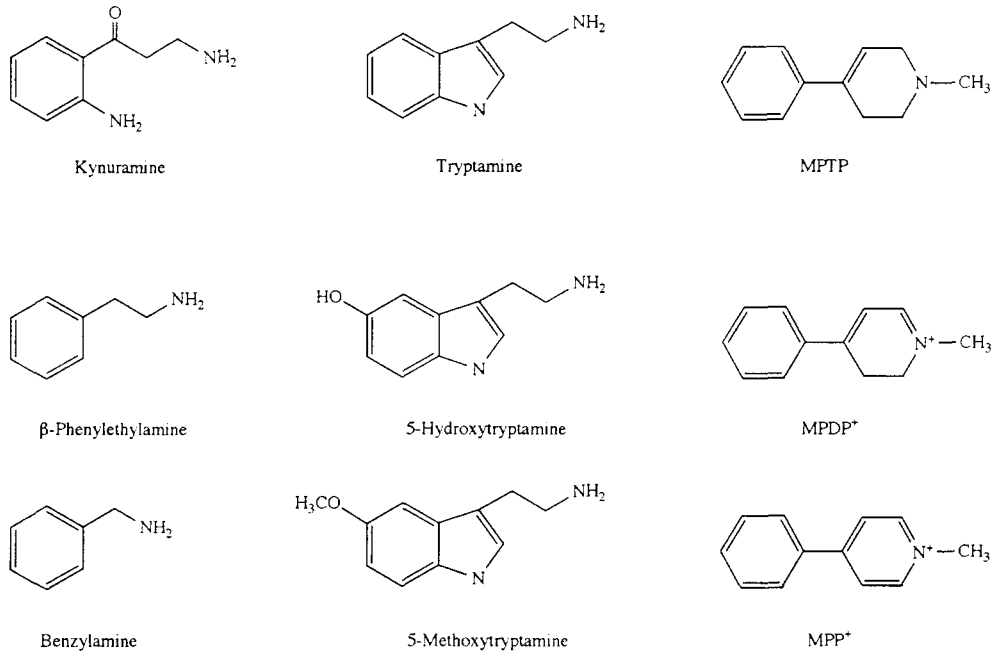


FIGURE 1: Structures of the ligands used.

obtained from Research Biochemicals Inc. Stock solutions of the neurotoxic agent, MPTP, and its derivatives were prepared and handled with caution as described before (Ramsay et al., 1987; Ramsay, 1991).

Enzymes. Monoamine oxidase A from human placenta (Weyler & Salach, 1985, 1987) and from human liver as overexpressed in yeast (Tan et al., 1991) and monoamine oxidase B from beef liver (Salach, 1979) were purified as reported previously. The enzyme was stored at −20 °C in a solution of 50 mM sodium phosphate, pH 7.2, 0.8% *n*-octyl β-D-glucopyranoside, 1.5 mM DTE, 0.5 mM D-amphetamine, and 50% glycerol. Before use, the enzyme was dialyzed against the appropriate buffer to remove DTE, D-amphetamine, and glycerol.

Stopped-Flow Experiments. The apparatus and procedure for anaerobic stopped-flow spectrophotometry were as described before (Ramsay et al., 1987; Ramsay, 1991). For the reductive half-reaction, the anaerobic enzyme solution was prepared in a tonometer and contained enzyme (16–20 μM flavin) in 50 mM sodium phosphate, pH 7.2, 30 mM glucose, glucose oxidase (1 unit/mL), and catalase (24 units/mL). For the oxidative half-reaction, the enzyme was reduced by equilibration with xanthine/urate reaction mediated by xanthine oxidase and methyl viologen (Ramsay, 1991). Methyl viologen (Paraquat), an analog of the product MPP^+, does not inhibit monoamine oxidase activity even at 2 mM. The amount used in the reduction is 10 μM.

Steady-State Kinetics. Initial rates of oxidation were measured in 50 mM phosphate, pH 7.2, with 0.2% Brij 35 at 30 °C. With kynuramine, the reaction was followed spectrophotometrically at 316 nm, with benzylamine at 250 nm, and with MPTP at 243 nm. The oxidation of all other substrates was measured polarographically.

RESULTS

Steady-State Kinetics. The steady-state kinetics for human MAO A from both liver and placenta and for beef liver MAO B with a variety of amine substrates (Figure 1) were studied at a fixed O_2 concentration (0.238 mM). For MAO A this concentration is well above the K_m value measured for placental MAO A [6 μM, measured with kynuramine or tyramine (Ramsay, 1991)] but for MAO B is about K_m [0.28 mM, measured with benzylamine (Husain et al., 1982)]. When oxygen is not saturating, the observed velocity still contains terms for the K_m for oxygen and the O_2 concentration, as do the apparent Michaelis constants obtained at saturating levels of amine.

$$V_{(app)} = \frac{V}{1 + \frac{K_m^B}{[B]}}$$

$$K_m^{A}{}_{(app)} = \frac{K_m^A\left(1 + \frac{K_{ia}K_m^B}{K_m^A[B]}\right)}{1 + \frac{K_m^B}{[B]}}$$

K_m^A and K_m^B are the Michaelis constants for amine and oxygen, respectively, and K_{ia} is the dissociation constant for the amine. Thus, for MAO B oxidizing benzylamine in air-saturated buffer at 30 °C, the $V_{(app)}$ is approximately half the true V_{max} and $K_m^A{}_{(app)}$ is about half the true K_m^A.

A full kinetic study of human liver MAO A, varying both amine and O_2, was done for kynuramine (Figure 2). The K_m for oxygen with the human liver form of MAO A was 60 ± 2.5 μM. A similar value was obtained with 5-hydroxytryptamine as the amine substrate (67 μM, data not shown). This K_m for oxygen is dramatically higher than the 6 μM found with the placental enzyme. Whether this difference is a fetal–adult adaptation or is a tissue specific feature which may be different in each tissue studied remains to be elucidated. This certainly suggests some difference in the oxygen reaction site which should be reflected in sequence differences between the liver and placental forms.

Half-Reactions of MAO A. Previous work used the enzyme from placenta (Ramsay, 1991). The data for both the reductive and oxidative half-reactions for the human liver enzyme are given in Table I. Although qualitatively similar, the rate constants obtained with the liver enzyme are generally higher than for the placental enzyme (e.g., k_3 for kynuramine

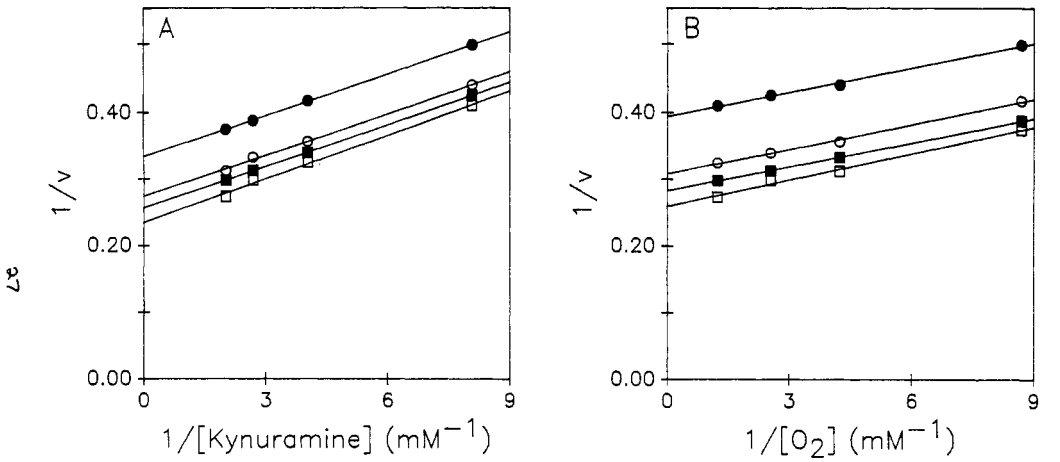


FIGURE 2: Steady-state kinetics for the oxidation of kynuramine for human liver MAO A. Assays were performed at 30 °C in 50 mM sodium phosphate, pH 7.2, and 0.2% Brij 35 and monitored spectrophotometrically at 316 nm. In panel A, the oxygen concentrations were 0.115 (●), 0.236 (○), 0.393 (■), and 0.806 mM (□). In panel B, the amine concentrations were 0.124 (●), 0.248 (○), 0.372 (■), and 0.496 mM (□).

Table I: Constants for the Reduction and Oxidation of Human Liver Monoamine Oxidase A

$$(1)\quad E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \xrightarrow{k_3} E_RP$$

$$(2)\quad E_RS + O_2 \xrightarrow{k_6} E_{ox}S + H_2O_2$$

	substrate		
constants[a]	MPTP	kynuramine[b]	5-HT[b]
reduction			
k_1 (M^{-1} s^{-1})	(5140)		
k_2 (s^{-1})	(0.26)		
k_3 (s^{-1})	0.15	3.1	2.1
K_a (mM)	0.08		
K_D (mM)	0.05	0.58	0.40
oxidation			
k_6 (mM^{-1} s^{-1})	166	508	24

[a] $K_a = (k_2 + k_3)/k_1$ and $K_D = k_2/k_1$. [b] Strickland plot was linear.

is 3.1 s^{-1} vs 1.7 s^{-1} for the placental enzyme). For all substrates, the reductive half-reaction gives rate constants which are very close to the turnover numbers in the steady state and much slower than the rate constants for reoxidation. Thus, for MAO A, the reductive half-reaction is rate-limiting with all substrates studied so far. No substrate has been found which reduces the enzyme faster than the overall rate of reaction.

The oxidative half-reaction reveals a wide variety of rates. As for the placental enzyme, free reduced liver MAO A is reoxidized only slowly (0.94 s^{-1} at air saturation), much slower than the steady-state turnover with the normal substrates. However, when the enzyme is preequilibrated with a substrate before mixing with oxygen in the stopped-flow chamber, the rate of oxidation is dramatically enhanced (Figure 3) as was observed for both MAO A from human placenta (Ramsay, 1991) and MAO B from beef liver (Husain et al., 1982; Ramsay et al., 1987). Even at the lowest concentration of ligand used (0.1 mM in Figure 3), the maximum stimulation was observed. The slopes in the presence of ligand in Figure 3 are the same and this was found for all the substrates tested. When the ligand is mixed with the enzyme at the same time as oxygen, rather than preincubated with the enzyme, no stimulation of the rate of oxidation is observed (Ramsay, 1991). Again, this was true for all the substrates tested here. These observations mean that both the rate of binding of the substrate to the reduced enzyme (k_1 in Scheme I) and the rate of dissociation of the E_R–S complex (k^1_2) are very much slower

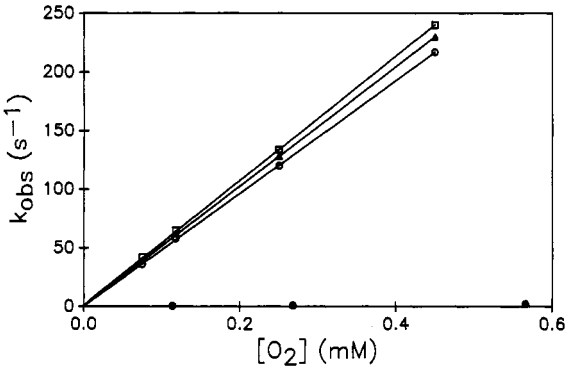


FIGURE 3: Dependence on oxygen concentration of observed rate constant for reoxidation of monoamine oxidase A alone or preincubated with kynuramine. The concentration of kynuramine were none (●), 0.1 (○), 0.5 (△), and 1.0 mM (□).

Table II: Kinetic Parameters for Human Liver MAO A from Steady-State and Stopped-Flow Half-Reaction Experiments

	steady state		reduction		oxidation	
substrate	k_{cat} (s^{-1})	K_m (mM)	k_3 (s^{-1})	K_D (mM)	k_{app} (s^{-1})[a]	k_{ox} (mM^{-1} s^{-1})
kynuramine	2.65	0.15	3.1	0.58	120	508
benzylamine	0.02	0.90	0.06	0.23	23	106
β-phenylethylamine	0.75	0.50	1.10	0.90	12	48
tryptamine	2.65	0.03	2.1	0.07	8	29
serotonin (5-HT)	2.80	0.40	2.1	0.40	5.7	24
5-methoxytryptamine	1.81	0.184	1.72	0.161	2.8	11
MPTP	0.20	0.09	0.16	0.07	40	166
$MPDP^+$	0.018	0.024	0.017	0.10	29	130
MPP^+					no reoxidation	
none					0.94	4.0

[a] At 0.238 mM O_2. All rates were measured at 30 °C.

than the rate of oxidation. Since the same (maximum) rate is obtained from 0.1–1 mM substrate (e.g., for kynuramine in Figure 3), the enzyme must all be in the E_RS form, even at the lowest concentration. Thus the K_D must be very small, but it cannot be calculated from data available. Table II shows that the enhancement of the rate of reoxidation varies with the substrate. Kynuramine (508×10^3 M^{-1} s^{-1}) enhances the oxidation rate by 125-fold, MPTP by 40-fold. The rest of the substrates used enhance the rate less than 25-fold. Benzylamine and β-phenylethylamine, although poor substrates for MAO A, give as good enhancement of the rate of reoxidation as 5-hydroxytryptamine, which is considered the best substrate for the A form of MAO.

Table III: Kinetic Parameters for Beef Liver MAO B from Steady-State and Stopped-Flow Half-Reaction Experiments

	steady state		reduction		oxidation	
substrate	k_{cat} (s^{-1})	K_m (mM)	k_3 (s^{-1})	K_D (mM)	k_{app} (s^{-1})[a]	k_{ox} (mM^{-1} s^{-1})[b]
kynuramine	2.75	0.084	13.6	1.11	2.17	2.89
benzylamine	10.0	0.36	10.9	0.14	7.6	29.4
β-phenylethylamine	3.62	0.067	572	4.5	1.8	5.35
tryptamine	0.67	0.13	0.63	0.15	2.1	5.45
5-hydroxytryptamine	0.077	0.28	0.097	0.24	1.7	4.12
5-methoxytryptamine	0.30	0.40	0.42	0.47	0.5	0.75
MPTP	0.16	0.04	0.12	0.06	6.0	23.3
MPP^+					no reoxidation	
none					1.3	5.49

[a] At 0.238 mM O_2. [b] The plots of K_{obsd} vs $[O_2]$ have nonzero intercepts, and the slope increases with the concentration of ligand, indicating that E_RS is reoxidized faster than E_R and that K_D^{ES} is high. Because the rates of oxidation and reduction of MAO B are similar, the experimental time courses describe the transition from fully reduced enzyme to a steady-state level of oxidation which is determined by the rate constants and the levels of amine and O_2 present. The approach to the steady state can be represented as

$$E_R \underset{g([S])}{\overset{f([O_2][S])}{\rightleftharpoons}} E_{ox}$$

and the observed rate takes the form

$$k_{obsd} = [k_{11} + k_{12}[S]/(K_D^{E_RS} + [S])][O_2] + k_{13}[S]/(K_m^s + [S])$$

where k_{11} is the bimolecular rate constant for the reoxidation which is given here as k_{ox}. The detailed derivation of this equation is given in Ramsay et al. (1987).

Modification of the indole ring of tryptamine has a strong influence on the rate of reoxidation (Table II), although the modification is remote from the amine group (Figure 1). Tryptamine yields a second-order rate constant for the reoxidation of the reduced enzyme–substrate complex (E_R–S) of 29×10^3 M^{-1} s^{-1}. Addition of a hydroxyl group at the 5 position (5-hydroxytryptamine, serotonin) decreases the rate constant slightly (24×10^3 M^{-1} s^{-1}), and addition of the electron-donating methoxy group decreases it more to 11×10^3 M^{-1} s^{-1}. The number of compounds studied is too small for general conclusions.

Half-Reactions of MAO B. The rates of the half-reactions of MAO B with benzylamine and MPTP (Husain et al., 1982; Ramsay et al., 1987) and for reduction with β-phenylethylamine (Husain et al., 1982) have been reported previously. For MPTP, the reductive half-reaction is rate-limiting. For benzylamine, the rate constants for the reductive and oxidative half-reactions are similar, so that both affect the overall rate of reaction. For β-phenylethylamine, the overall reaction is much slower than the reductive half-reaction, so that it was concluded that reoxidation was rate-limiting and that β-phenylethylamine was a true ping-pong substrate for MAO B (Husain et al., 1982). The parameters for the half-reactions of MAO B for these and additional amines are given in Table III. The reductive half-reaction with the indoleamines (normally used as substrates for MAO A) is very slow and corresponds to the rate of the overall reaction. Kynuramine is as good as a substrate for MAO B as is benzylamine on the basis of k_{cat}/K_m in air-saturated buffer. The reduction of MAO B by kynuramine (13.6 s^{-1}) is even faster than the rate with benzylamine, but the rate of the reoxidative half-reaction is slow. Thus, for MAO B with kynuramine, as with phenylethylamine, the oxidative half-reaction is rate-limiting, as was expected from the steady-state level of reduced enzyme in turnover experiments (Husain et al., 1982). The different between the K_D for kynuramine and the K_m measured by steady-state methods in air-saturated buffer emphasizes a long-known problem that the observed kinetic constants depend on the concentration of O_2 (Fowler & Oreland, 1980). If O_2 is depleted in the course of the reaction, erroneously low values for the apparent K_m would be observed and futher inhibition constants derived from them would be erroneous also. Thus, if simplified steady-state kinetic analysis is used, it is important to ensure that the substrate used in inhibition studies is one where the reductive half-reaction is rate-limiting.

The stopped-flow data from the oxidative half-reaction of MAO B are quite different from those obtained with MAO A. Plots of k_{obs} versus $[O_2]$ (Figure 5) are linear, but the slopes increase with substrate concentration and the intercepts are displaced from the enzyme. As discussed in earlier work

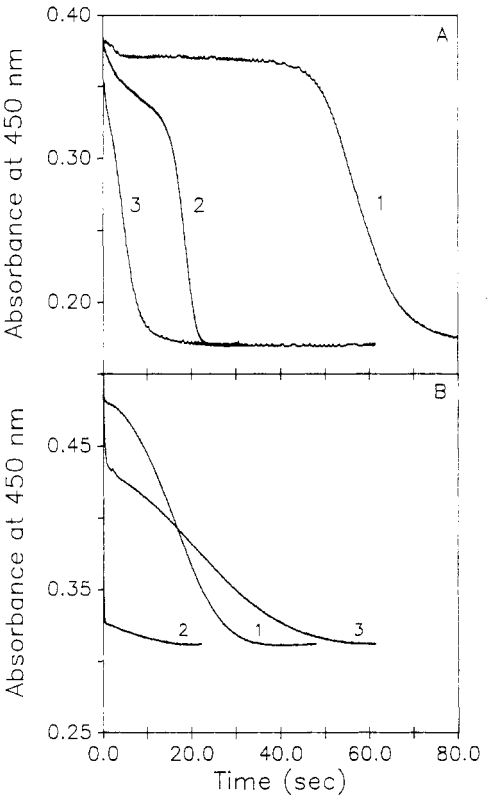


FIGURE 4: Redox state of the flavin in MAO A or B monitored during the turnover of various substrates. The enzyme was mixed in the stopped-flow spectrophotometer with equal volumes of buffer containing 10 mM substrate. The oxygen concentration was 0.238 mM. In panel A, MAO A (18 μM) was mixed with MPTP (curve 1), kynuramine (curve 2), or 5-hydroxytryptamine (curve 3). In panel B, MAO B (16 μM) was mixed with MPTP (curve 1), 5-methyoxytryptamine (curve 2), or 5-hydroxytryptamine (curve 3).

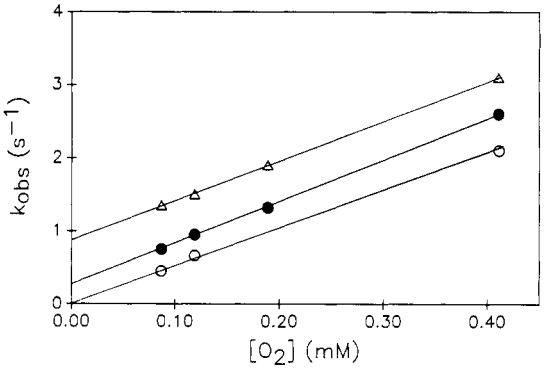


FIGURE 5: Dependence on oxygen concentration of observed rate constant for reoxidation of monoamine oxidase B alone or preincubated with β-phenylethylamine. The concentration of β-phenylethylamine were none (○), 0.26 (●), or 2.0 mM (△).

Table IV: Enhancement of Reoxidation of Monoamine Oxidase A Does Not Correlate with Rate of Reduction

substrate	k (s^{-1}) reduction	oxidation
5-hydroxytryptamine	2.1	5.7
MPTP	0.20	40
$MPDP^+$	0.02	29

(Ramsay et al., 1987), these patterns arise from the form of the function describing the oxidative step on which is superimposed reduction of the oxidized enzyme by the excess substrate present.

The data were fitted to

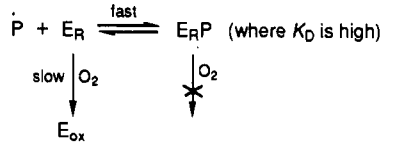
$$k_{obs} = [k_{11} + k_{12}[S]/(K_D^{E_RS} + [S])][O_2] + k_{13}[S]/(K^s_m + [S])$$

to obtain the values given in Table IV. However, the derivation of the equation makes the rapid equilibrium assumption, which is not valid for those substrates which show curvature in the double-reciprocal plots for the reductive half-reaction (e.g., benzylamine). (The curvature indicates that k_2 and k_3 have similar values, and thus the binding of substrate to the oxidized enzyme will not be at equilibrium in the reductive half-reaction experiments.) For the reduced enzyme, the on rate for substrate binding must be slower than the reoxidation (k_4) because substrate mixed with the enzyme at the same time as oxygen has no effect on k_4. Thus K_D for the E_RS complex cannot be determined. Further, it cannot be assumed that K_D^{ERS} is the same as K_{DEoxS}. Indeed, for β-phenylethylamine, the only substrate where the rapid equilibrium assumption is valid at least in the reductive half-reaction, the K_D values may differ by an order of magnitude. When the data from Figure 4 are fitted to the equation above, k_{11} and k_{12} are 2.51×10^3 M^{-1} s^{-1} and 7.34×10^3 M^{-2} s^{-1}, respectively. Their ratio, an estimate of K_D^{ERS}, is 0.32 mM compared to the K_D from Table III of 4.5 mM.

Table III reports for the first time the rate of reoxidation in the presence of β-phenylethylamine. The second-order rate constant is essentially the same as that measured for reoxidation of the free enzyme (5500 M^{-1} s^{-1}). The highest amount of β-phenylethylamine used was about 6-fold higher than the estimated K_D so that approximately 84% of the enzyme would be in the E_RS form. High concentrations of β-phenylethylamine (2 mM) mixed with the enzyme at the same time as oxygen also gave no effect. Providing the estimate of K_D^{ERS} is reasonable, the data presented suggest that E_R–PEA is reoxidized at the same rate as free enzyme, so no effect on the steady-state kinetics can be expected.

Benzylamine and MPTP stimulate the rate of reoxidation by 5- and 4-fold, respectively [Ramsay et al. (1987); Table III]. For MPTP, this is a much more modest enhancement than observed with MAO A (40-fold, Table II), despite the fact that, in the absence of substrate, the free reduced enzymes are reoxidized at similar rates (4000 M^{-1} s^{-1} for MAO A and 5500 M^{-1} s^{-1} for MAO B). The indoleamines enhance the reoxidation of MAO B by only 1.5–3-fold.

MPP^+, the ultimate product of the two-step oxidation of MPTP, is a competitive inhibitor of the steady-state oxidation of amines by MAO (Singer et al., 1985). In the steady-state assay, free reduced enzyme cannot be detected by the kinetic effect of inhibition by amphetamine (Pearce & Roth, 1985; Ramsay, 1991), which has a much lower K_i than does MPP^+, so we tested the effect of MPP^+ on the reoxidation of enzyme reduced by the xanthine oxidase method. MPP^+ was found to completely inhibit the stimulation of reoxidation of placental MAO A by traces of kynuramine, but reoxidation at the same rate as free enzyme was still observed, suggesting that the E_R–MPP^+ complex (E_R–P) either was not reoxidized or was reoxidized at the same rate as free enzyme. In further experiments, we have now been able to show the inhibition of oxidation by MPP^+. The simplest explanation of the variability is that MPP^+ binds poorly to E_R in contrast to its tight binding to E_{ox} (K_i = 4 μM) so that high concentrations must be used to obtain sufficient E_R–P to observe a decrease in the slow rate of reoxidation of free E_R.



Similarly for substrate, the preliminary computer modeling runs based on the data in Table II predict a higher K_D for E_RS than for $E_{OX}S$ in order to obtain the turnover curves shown in Figure 4. We conclude that our original proposal stands: the flux through the alternate paths of reoxidation of free reduced enzyme or the reduced enzyme–substrate complex is determined by the relative dissociation constants and rate constants. MAO catalysis is thus flexible with varying amine and oxygen levels.

Stopped-Flow Monitored Turnover. Turnover experiments where enzyme is mixed with a high concentration of substrate but limiting O_2 can be used to determine the redox state of the flavin during steady-state turnover. Figure 4 shows the time courses for the reduction of the flavin of MAO A by excess substrate. Clearly, the redox state of the flavin depends on the substrate. Where the enzyme remains completely oxidized during turnover, for example with MPTP, the ratio for the oxidative to reductive rate constants (k_{app}/k_3) from Table II is greater than 50. The same is observed with MAO B. In contrast, the substrates which give more reduced flavin in the steady-state, such as β-phenylethylamine for MAO B (Husain et al., 1982), have ratios of less than 1. Intermediate ratios, such as for kynuramine (39) or 5-hydroxytryptamine (2.7) with MAO A, correlate with intermediate patterns of reduction of the flavin (Figure 4). With kynuramine, the flavin in MAO A remains 95% oxidized at the onset of the steady state, whereas with 5-hydroxytryptamine it is 78% oxidized. Further, although both substrates are oxidized at the same steady-state rate (Table II) the redox state of the flavin changes quite differently with the time for the two substrates. The lower the k_{app}/k_3 ratio, the faster the reduced enzyme species accumulate. Since reoxidation is a bimolecular reaction between oxygen and the reduced enzyme (E_RP or E_RS), the steady-state level of reduced enzyme will affect the observed rate of reoxidation.

DISCUSSION

Previous papers from this laboratory demonstrated that, contrary to the generally held views at the time, the reoxidation of MAO involved competing pathways via free or liganded reduced enzyme. Further, the ternary complex which yielded the fastest rate of reoxidation of reduced MAO was not the complex with product but the complex with substrate, E_R–S–O_2. The importance of this becomes evident in the present study which indicates that the rate of the ternary complex pathway is dependent on the nature of the substrates. That this pathway is important under turnover conditions is apparent

from Figure 4, which indicates that the steady-state levels of oxidized reduced enzyme depend on the substrate and its efficacy in stimulating the reoxidative half-reaction. The practical implication is that complex kinetics may be observed for this enzyme. Current studies in our laboratory have demonstrated that the oxidation of tetrahydrostilbazoles yield biphasic kinetic plots as a consequence of the branched mechanism (R. R. Ramsay, S. O. Sablin, S. O. Bachurin, and T. P. Singer, to be published). At low concentrations of substrate, the reoxidation via the free enzyme predominates whereas at high substrate concentrations the faster ternary complex pathway determines the steady-state rate. Although normal double-reciprocal plots are obtained from the substrates studied here, the differences in the steady-state redox state of the enzyme with different amines and potentially different inhibitor binding characteristics for the oxidized and reduced enzyme mean that simple inhibition studies must be analyzed with caution.

The survey of the half-reaction rate constants for a series of primary and tertiary amine substrates with both human liver MAO A and beef liver MAO B (Tables II and III) leads to several conclusions about the effect of substrate on the catalytic reaction. For MAO A, unlike MAO B, no substrate was found for which reoxidation of the reduced enzyme in the presence of substrate was slower than the reduction. Paradoxically, the normal "A" substrates such as kynuramine, serotonin, and tryptamine exhibit reductive rate constants of about 2.6 s^{-1} (Table II) which are greater than the rate of reoxidation of the free reduced enzyme (0.9 s^{-1}). However, the rate of reoxidation of reduced enzyme preequilibrated with substrate is dramatically faster than reoxidation of the free enzyme. The enhanced oxidative rate constants (Table II, column 7) fall into two groups. The first group has the super-fast rates obtained with kynuramine and MPTP as previously reported for placental MAO A (Ramsay, 1991). The second group contains the substrates generally held to be "natural" substrates of MAO A in vivo, such as 5-hydroxytryptamine and tryptamine, but also includes the "B" substrates, β-phenylethylamine and benzylamine. Each substrate in this group stimulates the reoxidation by a factor of only about 6-fold. There is no immediately obvious distinguishing difference between the structures represented in two groups (see Figure 1) nor any feature shared by MPTP and kynuramine which could explain this difference. Presumably, the explanation must lie in the particular fit of the substrate within the active site in relation to binding groups (hydrogen-bonding groups or hydrophobic areas) or to the magnitude of a consequent conformational change which alters the reaction with oxygen. Understanding the factors involved may have to await a picture of the structure of the active site from X-ray crystallography.

$MPDP^+$ is a special case, the oxidation rate constant being only slightly less than that for MPTP. It is unique in being both a product and a substrate. It reduces MAO A only slowly (Table I), at a tenth of the rate of MPTP which in turn reduces MAO A 10-fold slower than does kynuramine. However, both MPTP and $MPDP^+$ stimulate the reoxidation far better than do the indole substrates, the enhancement of the oxidative rate constant by MPTP being 40-fold, by $MPDP^+$ being 30-fold. Thus, $MPDP^+$ is only slightly less effective than MPTP at stimulating the reaction with oxygen, which must be related to its increased unsaturation since it differs from MPTP by only two hydrogen atoms. The product, MPP^+, inhibits the reoxidation. These results strongly suggest that the ability to denote electrons to the flavin is important. However, there is no correlation between the rate of reduction and the rate of reoxidation of the E_R–S complex (Table IV), so additional factors must influence the efficacy.

When the kinetics of the steady-state and half-reactions of MAO B are examined with the same substrate quite a different picture from that for MAO A emerges. Although, again, all the substrates stimulate the reoxidative half-reaction, the stimulation is 10–15-fold, and, for some substrates, this enhanced rate is still slower than the rate of reduction (Table I). The oxidative half-reaction is rate-limiting for β-phenylethylamine (Husain et al., 1982) and for kynuramine and must affect the steady-state rate for benzylamine (Ramsay, 1991). The indole substrates are both slow reductants for MAO B and stimulate the reoxidation poorly. However, with MAO B, in contrast to MAO A, kynuramine gives only the same stimulation of reoxidation as do the indole substrates. Clearly, the substrate site of MAO B is unable to provide the favorable interaction between kynuramine and reduced flavin that occurs in MAO A.

The notion of identical flavin sites in the A and B forms, with different substrate sites, now seems inappropriate. The substrate site is closely related to the flavin not just for its reduction but in affecting the interaction of the flavin with oxygen. Substrate or product binding is known to induce changes in flavin enzymes. For example, binding of various CoA derivatives markedly slows the reoxidation of reduced acyl-CoA dehydrogenases by oxygen (Beinert, 1963; Wang & Thorpe, 1991). For the medium-chain acyl-CoA dehydrogenase, reduced enzyme and product form a charge transfer complex which is less reactive with oxygen than the free enzyme (Beinert, 1963). A reduced enzyme–product charge-transfer complex has also been characterized for various oxidases, for example, L-phenylethylamine oxidase (Suzuki et al., 1991). It has also been observed that there is a dramatic shift in the midpoint potential for the general acyl-CoA dehydrogenase when acyl-CoA is bound which favors the transfer of electrons from substrate to enzyme (Lenn et al., 1990). Further, structural differences between classes of flavoproteins are reflected in different oxygen reactivities (Massey et al., 1988). The enhancement of the rate of oxidation of the MAO flavin by oxygen could result from small conformation changes transmitted perhaps to the flavin or perhaps to the oxygen binding site to stabilize the oxygen intermediates. Some aspects of the solvation of oxygen and the stabilization of the intermediates in its reduction to H_2O_2 have been discussed by Wang and Thorpe (1991) in relation to the decreased reactivity of acyl-CoA dehydrogenases and increased reactivity of acyl-CoA oxidases when product is bound to the enzyme. Whatever the mechanism of the enhancement of MAO reactivity with oxygen, the data here indicate that the structure of A site is more effective than that of the B site. Without ligand, both the A and B forms are reoxidized at the same rate. When a given substrate is bound, the enhancement of the reoxidation rate is greater for the A form, so one or more of the groups involved in the effect must differ in type or orientation from that in the B form. These subtle differences represents a new avenue for the investigation of the oxygen reactivity of flavoproteins.

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