Stopped-Flow Studies on the Mechanism of Oxidation of *N*-Methyl-4-phenyltetrahydropyridine by Bovine Liver Monoamine Oxidase B†

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ABSTRACT: The kinetic mechanism of monoamine oxidase B involves either a binary or a ternary complex, depending on the substrate. In this study, stopped-flow kinetic data provide direct evidence for ternary complexes not only of reduced enzyme, oxygen, and product but also of reduced enzyme, oxygen, and substrate, both for benzylamine and for the tertiary amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). However, the mechanism for a given substrate is not exclusive but, rather, is determined by competition between the alternate pathways as a result of different rate constants for the oxidation of the reduced enzyme, the reduced enzyme–product complex, and the reduced enzyme–substrate complex, as well as the different dissociation constants for the complexes. Comparison of the rate constants obtained from the stopped-flow studies with steady-state data indicates that the overall rate of reaction for the oxidation of MPTP by monoamine oxidase is dominated by the reductive step, but for benzylamine the steady-state rate is determined by a complex function of the rates of both the reductive and oxidative half-reactions.

In earlier studies in this laboratory (Husain et al., 1982), it was concluded that the kinetic mechanism of monoamine oxidase B depends on the nature of the substrate, as is true with some other flavoenzymes (Bright & Porter, 1975). The results of steady-state and pre-steady-state experiments, including measurements of kinetic isotope effects, suggested that the relative rates of the dissociation of the product from E_R–P, the reduced enzyme–product complex, and the interaction with O_2 determined whether a binary (ping-pong) or ternary mechanism occurred. Among the substrates tested, (phenylethyl)amine seemed to be oxidized by a purely binary mechanism, whereas with benzylamine a ternary complex mechanism operated. These conclusions were recently confirmed by Pearce and Roth (1985) in elegant steady-state experiments.

The fact that the products formed from benzylamine and (β-phenylethyl)amine [benzylimine and (β-phenylethyl)imine, respectively] are rapidly hydrolyzed limited mechanistic studies with these substrates. An opportunity to further probe the kinetic mechanism of monoamine oxidase B arose when it was found (Salach et al., 1984; Singer et al., 1986) that the neurotoxic tertiary amine MPTP[1] was rapidly oxidized by the enzyme to $MPDP^+$, the dihydropyridinium species. The latter is sufficiently stable to be added to the reduced enzyme, permitting stopped-flow studies on the influence of product concentration on the oxidative half-reaction. $MPDP^+$ has been shown to be a good competitive inhibitor of monoamine oxidase B and to be slowly oxidized to MPP^+, the *N*-methyl-4-phenylpyridinium species (Singer et al., 1985, 1986a).

Studies on the kinetic mechanism of oxidation of MPTP by monoamine oxidase B are also of interest to enzymologists because the rapid oxidation of a tertiary amine by the enzyme had been unexpected and to neurochemists because the action of monoamine oxidase on MPTP is the initial step in its neurotoxicity, leading to the development of experimental Parkinsonism (Singer et al., 1986a,b). This paper shows that the dominant step in determining the rate of oxidation of both MPTP and $MPDP^+$ is the reductive half-reaction and suggests that there are three competing pathways for the reoxidation of the reduced enzyme.

Materials and Methods

Reagents. Benzylamine (Sigma) was recrystallized from ethanol before use. MPTP (Aldrich) and $MPDP^+$ (Chiba et al., 1985) were obtained as in previous work (Singer et al., 1986a). Stock solutions of $MPDP^+$ (20 mM) were prepared in water adjusted to pH 2 to prevent disproportionation. Because of the neurotoxicity of these compounds, isolation precautions were taken throughout this work.

Enzyme. Monoamine oxidase was purified from beef liver mitochondria and assayed as described by Salach (1979). The specific activity was 4.4 units/mg of protein, and the enzyme was homogeneous by the usual criteria. It was stored at −20 °C in 50% glycerol, at a concentration of 17.4 mg of protein/mL, and 154 μM flavin. Before use, glycerol was removed by diluting an aliquot 20-fold in 50 mM NaP_i, pH 7.0, and sedimenting the enzyme at 198000*g* for 30 min. The pellets were suspended in 50 mM Hepes, pH 7.5, containing 1% Triton to reduce turbidity. In this form, the enzyme may be kept on ice for several days.

Stopped Flow. Anaerobic stopped-flow spectrophotometry was carried out on a system from Kinetic Instruments, Inc. (Ann Arbor, MI). The photomultiplier signal was fed through an Amico current-to-voltage transducer into an IBM-PC compatible set of hardware and software systems. Rapid data

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium species.

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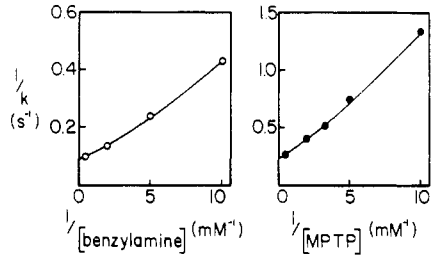
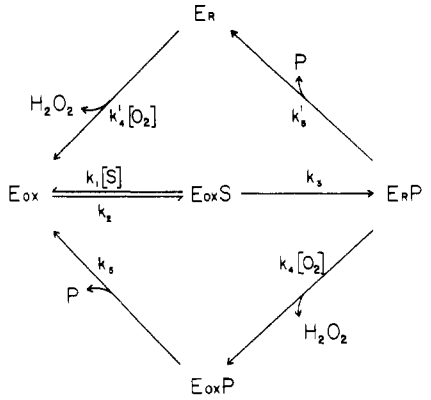


FIGURE 1: Dependence of observed rate constant for reduction of monoamine oxidase on substrate concentration. The method is described under Materials and Methods. The reaction mixture contained 6.1 μM flavin, 50 mM Hepes, pH 7.5, 0.5% Triton X-100, 30 mM glucose, 1 unit of glucose oxidase, 24 units of catalase, and substrate as indicated in the double-reciprocal plots.

Scheme I



acquisition and its analysis were as described previously (Ramsay et al., 1987). The stopped-flow apparatus was prepared for anaerobiosis by overnight incubation with a solution of 50 μM riboflavin reduced with 1 mM EDTA and light. Anaerobic 50 mM Hepes, pH 7.5, containing 30 mM glucose, 1 unit/mL glucose oxidase and 24 units/mL catalase was used to flush the syringes and chamber before use. The temperature was 25 °C.

For the reductive half-reaction the anaerobic enzyme solution was prepared in a tonometer and contained enzyme (12.2 μM flavin) in 50 mM Hepes, pH 7.5, 30 mM glucose, glucose oxidase (1 unit/mL), and catalase (24 units/mL).

In the studies of the oxidation half-reaction, reduced monoamine oxidase B was prepared by anaerobic titration with either benzylamine or MPTP. The oxygen concentration was varied by mixing different volumes of anaerobic and oxygen-flushed buffer (50 mM Hepes, pH 7.5). The resulting oxygen concentration was measured polarographically with a Clark oxygen electrode.

RESULTS

Reductive Half-Reaction. The rate constants for the reduction of monoamine oxidase B by benzylamine or MPTP, determined by anaerobic stopped-flow spectrophotometry, are shown in Figure 1. These data were analyzed by a modification of the method of Strickland et al. (1975). Scheme I, modified from Bright and Porter (1975) and Husain et al. (1982), shows two alternative kinetic mechanisms.

In the reductive half-reaction

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

the oxidized enzyme (E_{ox}) complexes with the substrate to form the enzyme–substrate complex (E_{ox}·S) and then is reduced to the enzyme–product complex (E_R–P). The reduction step is accompanied by an absorbance change and is characterized by the rate constant k_3. If [S] > [E_{ox}], the production of E_R–P will be a biphasic process

$$[E_RP]/[E_{total}] = 1 + A_\alpha e^{-k_\alpha t} + A_\beta e^{-k_\beta t} \qquad (2)$$

where $k_\alpha = (p + q)/2$, $k_\beta = (p - q)/2$, $p = k_1[S] + k_2 + k_3$, $q = (p^2 - 4k_1k_3[S])^{1/2}$, $A_\alpha = (k_\alpha/k_\beta - 1)^{-1}$, $A_\beta = A_\alpha + 1$, and A_α and A_β are the amplitudes of the slow and fast steps, respectively (Hiromi, 1979).

The amplitude A_α, representing the absorbance change as a result of substrate binding, is small, introducing only a minor perturbation in the analysis of the stopped-flow time courses, and the data fit well to monophasic exponentials (data not shown). Thus, the observed rate

$$k_{obsd} = k_\beta = k_1[S] + k_2 + k_3 - [(k_1[S] + k_2 + k_3)^2 - 4k_1k_3[S]]^{1/2} \qquad (3)$$

or

$$1/k_{obsd} = 2(1/[S])/\{k_1 + (k_2 + k_3)(1/[S]) - [[k_1 + (k_2 + k_3)(1/[S])]^2 - 4k_1k_3(1/[S])]^{1/2}\} \qquad (4)$$

When the data are plotted by the method of Strickland et al. (1975), using eq 4, the reciprocal plot is concave upward. The data were fit to eq 4 according to a nonlinear least-squares method (Bevington, 1969), based on the gradient expansion algorithm of Marquardt (1963).

Table I: Constants for Reduction of Monoamine Oxidase B by Benzylamine and MPTP: $E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \xrightarrow{k_3} E_RP$

constants[a]	substrate: benzylamine	substrate: MPTP
k_1 (M^{-1} s^{-1})	37000 ± 200	7100 ± 200
k_2 (s^{-1})	5.4 ± 0.8	0.26 ± 0.09
k_3 (s^{-1})	10.9 ± 0.2	3.7 ± 0.2
K_a (mM)	0.432	0.558
K_D (mM)	0.143	0.037

[a] $K_a = (k_2 + k_3)/k_1$ and $K_D = k_2/k_1$. Experimental details are given in Figure 1.

The rate constants obtained by this analysis are shown in Table I. As predicted by the curvature of the double-reciprocal plots (Figure 1), k_2 and k_3 are comparable in value for each substrate but k_3 for benzylamine is almost 3 times greater than k_3 for MPTP. These values for k_3 are close to the turnover numbers obtained by steady-state methods (see Discussion).

Oxidative Half-Reaction. Reoxidation by O_2 of monoamine oxidase B that was reduced with a slight excess of substrate is a simple bimolecular process with both benzylamine and MPTP (Figure 2, lower line), as previously reported for the former substrate (Husain et al., 1982). The second-order rate constants from this experiment were 16.8 × 10^3 M^{-1} s^{-1} and 17.1 × 10^3 M^{-1} s^{-1} for benzylamine and MPTP, respectively. Both values are lower than those observed in the presence of an excess of these substrates (see below) but significantly higher than the rate constant of 6 × 10^3 M^{-1} s^{-1} previously reported for the oxidation of the dithionite-reduced enzyme (Husain et al., 1982). The enzyme preparation used in this work was considerably purer than that used in the experiments quoted and could not be reduced with dithionite beyond the semiquinone stage.

It has been proposed that the greater rate of reoxidation of the substrate-reduced enzyme than of the dithionite-reduced enzyme is the result of the formation of a ternary complex between reduced enzyme, product, and O_2 (Husain et al., 1982; Pearce & Roth, 1985). Implicit in this hypothesis is that the rate may vary with the nature of the product. This inter-

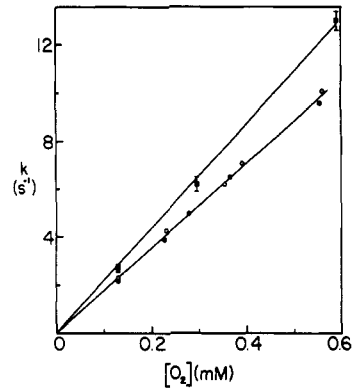


FIGURE 2: Apparent second-order plots for reoxidation of monoamine oxidase. Monoamine oxidase (4.4 μM flavin) was reduced with a 2-fold excess of benzylamine (○) or MPTP (●). In a separate experiment, monoamine oxidase (5.3 μM flavin) was reduced with a 4-fold excess of MPTP, and excess MPDP (20–500 μM) was added (■). The rate of oxidation was independent of MPDP concentration. Other conditions are described in Figure 1 and under Materials and Methods.

pretation could not be proven directly with substrates previously used, because of rapid hydrolysis of the corresponding imines, but $MPDP^+$, the two electron oxidation product of MPTP, is sufficiently stable to test this hypothesis. The oxidative half-reaction was therefore studied at various concentrations of the two substrates (benzylamine and MPTP) and product ($MPDP^+$) to see if the different ternary complexes are indeed oxidized at different rates.

In the presence of all concentrations of the product, MPDP (0.02–0.50 mM), a simple second-order reaction was observed (Figure 2), as with the substrate-titrated enzyme. The second-order rate constant was $(21.8 \pm 0.37) \times 10^3$ M^{-1} s^{-1}.

In the presence of excess of either substrate, the analysis is more complicated. The time courses characterizing the oxidative half-reaction were monophasic exponentials representing the transition from fully reduced enzyme at zero time to some steady-state value determined by the concentrations of O_2 and substrate. Had the reaction been allowed to proceed, the final concentration of reduced enzyme would be determined by the reagent in excess. The simplest scheme describing this approach to the steady-state is given by

$$E_R \underset{g([S])}{\overset{f([O_2],[S])}{\rightleftharpoons}} E_{ox} \qquad (5)$$

where the observed rate is the sum of the forward and reverse rates, shown as the functions $f([O_2],[S])$ and $g([S])$.

Previous work (Husain et al., 1982) and the current studies show the reaction of O_2 with reduced enzyme to be a simple bimolecular process with no indication of saturation within the observable concentration range (Figure 2). However, as will be shown, the function $f([O_2],[S])$ is complicated by the possibility of the reduced enzyme–substrate complex (E_R–S) reacting with O_2 at a rate different from the reaction of E_R with O_2 (Scheme II). Consequently, we assumed the forward rate to have the form

$$f([O_2],[S]) = [k_{11} + (k_{12}[S]/K_D^{E_RS} + [S])][O_2] \qquad (6)$$

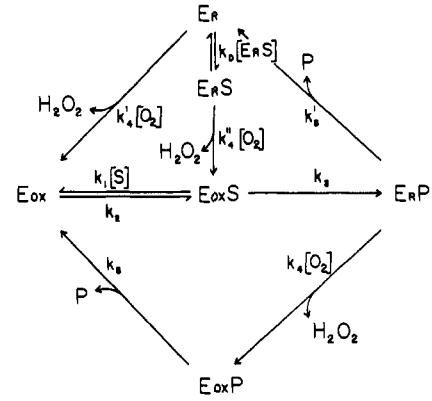
The reverse reaction $g([S])$ is the simple reduction of oxidized enzyme by substrate and has the form

$$g([S]) = k_3[S]/(K_D^{E_{ox}S} + [S]) \qquad (7)$$

Thus, the observed rate has the form

$$k_{obsd} = [k_{11} + k_{12}[S]/(K_D^{E_RS} + [S])][O_2] + k_{13}[S]/(K_M^S + [S]) \qquad (8)$$

Scheme II



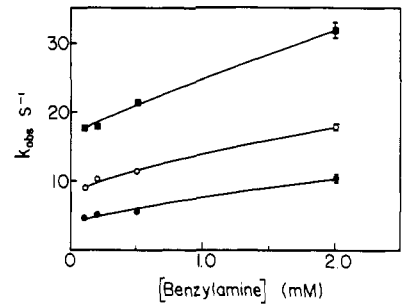


FIGURE 3: Effect of benzylamine on reoxidation of reduced monoamine oxidase. The flavin concentration was 4.6 μM. Benzylamine (0.1–2 mM) was added to the enzyme solution. The final oxygen concentration was 0.129 mM (●), 0.286 mM (○), or 0.57 mM (■). Details are given under Materials and Methods. The points are averages of at least five determinations. The error bars indicate ± standard deviation, but in most cases the deviation was less than the size of the point.

At fixed $[O_2]$, the k_{obsd} vs. [S] plots are curved for benzylamine and even more so for MPTP (Figures 3 and 5, respectively). Further analysis was not attempted because the term causing the curvature

$$k_{13}[S]/(K_M^S + [S])$$

makes the rapid equilibrium assumption, which the results of the reductive half-reaction show not to hold.

At a fixed [S], k_{obsd} is a linear function of $[O_2]$ (Figures 4A and 6A), with slope = $k_{11} + k_{12}[S]/(K_D^{E_RS} + [S])$ and intercept = $k_{13}[S]/(K_M^S + [S])$.

The observation that, for both benzylamine (Figure 4B) and MPTP (Figure 6B), the slope of k_{obsd} vs. $[O_2]$ increases with [S] is prima facie evidence that there is a specific E_R·S complex that reacts with O_2 faster than does E_R. The limiting value of the slope as $[S] \rightarrow 0$ is the bimolecular rate constant k_{11}. The values are 29.4×10^3 M^{-1} s^{-1} for benzylamine and 23.3×10^3 M^{-1} s^{-1} for MPTP (k_{11} in Table II). There is no evidence with either substrate for saturation of the slope with [S]. We take this to indicate that $K_D^{E_RS}$ is very large with respect to the [S] used in these studies. Consequently, the only information available is the ratio $k_2/K_D^{E_RS}$, which is the slope of the slope replot (Figure 4B for benzylamine and Figure 6B for MPTP).

The rate constants shown in Table II were obtained by fitting all the data for the reoxidative half-reaction to a function of both the variables, [S] and $[O_2]$. The values for k_{11} reflect the reoxidation of all reduced species of enzyme (E_R + E_R–S + E_R–P). The hybrid rate constant k_{12}^* represents $k_{12}/(K_D^{E_RS} + [S])$ (see eq 8), which approximates to $k_{12}/K_D^{E_RS}$ when $K_D^{E_RS} \gg [S]$, as is indicated by the lack of saturation in the k_{obsd} vs. $[O_2]$ plots. The values for k_{13} should be close

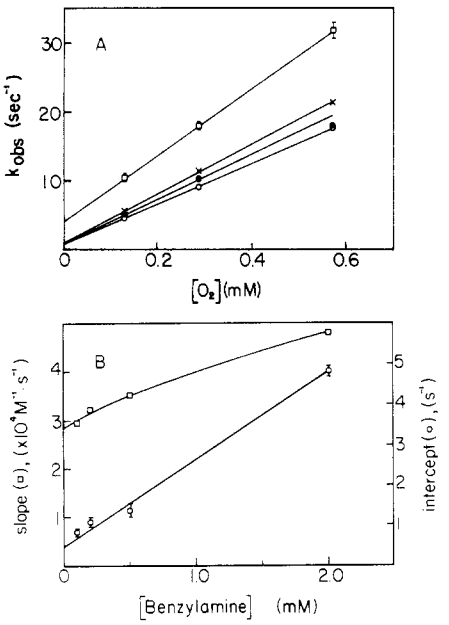


FIGURE 4: Dependence on oxygen concentration of observed rate constant for reoxidation of monoamine oxidase in the presence of excess benzylamine. The data come from the same experiment as shown in Figure 3. (A) The final benzylamine concentration was 0.1 (○), 0.2 (●), 0.5 (■), or 2 mM (□). (B) Secondary plot of the slopes (□) and intercepts (○) from (A).

Table II: Rate Constants for Oxidation of Monoamine Oxidase in the Presence of Excess Substrates: $E_R \underset{g([S])}{\overset{f([O_2],[S])}{\rightleftharpoons}} E_{ox}$

	substrate	
constants[a]	benzylamine	MPTP
k_{11} (M^{-1} s^{-1})	(2.94 ± 0.07) × 10^4	(2.33 ± 0.04) × 10^4
k_{12}* (M^{-2} s^{-1})	(9.63 ± 0.79) × 10^6	(3.92 ± 0.39) × 10^6
k_{13} (s^{-1})	6.98 ± 2.04	7.82 ± 0.75
K_{14} (mM)	1.30 ± 0.70	0.98 ± 0.19

[a] The complex constants are designated in $k_{obsd} = (k_{11} + k_{12}^*[S])[O_2] + k_{13}[S]/(K_{14} + [S])$. Experimental details are given in Figures 3 and 5. Note that k_{11} in this table is derived from a computer fit of the experimental data and may not represent k_4, the rate of reoxidation of E_R, if the curve becomes biphasic at very low concentrations of substrate.

to those obtained in the reductive half-reaction since this is what the term

$$g([S]) = k_3[S]/(K_D^{E_{ox}S} + [S]) \quad (9)$$

describes. However, because the rapid equilibrium assumption made in the derivation is not valid (as discussed above), it is not surprising that these values (k_{13} and k_3) differ. The values for k_{13} for benzylamine and MPTP are similar, which suggests that contributions from the different on and off rates for the different substrates and products modify the k_3 observed in the reductive half-reaction.

Similarly, K_{14} may not represent the true K_M^S.

Discussion

Studies of the kinetic mechanism of glucose oxidase (Bright & Porter, 1975) and of monoamine oxidase B (Husain et al., 1982) by steady-state and pre-steady-state methods established that certain flavoenzymes may follow either a binary or a ternary complex mechanism, depending on the substrate being processed. This was visualized as the result of a kinetic competition between product dissociation from E_R–P and the reaction of this complex with O_2 represented by k_5' and k_4 in Scheme I. In this scheme the upper loop represents the binary complex mechanism proposed for the oxidation of (phenylethyl)amine, tyramine, and tryptamine by monoamine oxidase B, while the lower loop is the ternary complex mechanism which has been proposed to function exclusively in the oxidation of benzylamine by the enzyme (Pearce & Roth, 1985; Husain et al., 1982).

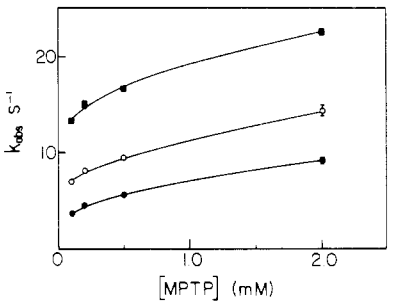


FIGURE 5: Effect of MPTP on reoxidation of reduced monoamine oxidase. The flavin concentration was 4.6 μM. MPTP (0.1–2 mM) was added to the enzyme solution. The final oxygen concentration was 0.129 (○), 0.282 mM (●), or 0.56 mM (■). Details are given under Materials and Methods. The points are averages of at least five determinations. The error bars indicate the standard deviation, but in most cases the deviation was less than the size of the point.

It may be noted that the papers quoted assume the existence of an unique kinetic mechanism that is characteristic of the chemical nature of the product present in E_R–P, the reduced enzyme–product complex. This is explicitly stated by Pearce and Roth (1985), who speculated on the reasons why a ternary complex mechanism occurs uniquely during benzylamine oxidation and why E_R "the free reduced form of the enzyme does not exist...". They suggested that either the product may uniquely distort the flavin ring system, which brings about an increase in the rate of its reoxidation, or that resonance stabilization of the E_R–benzylimine complex by conjugation with the aromatic ring of the flavin may increase its stability.

The data presented above show that benzylamine is, in fact, not unique in forming a ternary complex with monoamine oxidase B, since oxidation of the structurally dissimilar tertiary amine MPTP by monoamine oxidase B also occurs by a ternary complex mechanism. Moreover, our data suggest that the kinetic mechanism that operates with any given substrate of monoamine oxidase B is not exclusively a binary or ternary complex mechanism but instead a "mechanism spectrum" may exist, ranging from a predominantly binary complex to a predominantly ternary complex, depending on the substrate, and that, in fact, the ternary complex may involve not only E_R–P–O_2 but also E_R–S–O_2 (Figures 3 and 5). A revised scheme (Scheme II) is offered to help visualize these possibilities.

In this scheme a new complex (i.e., E_R–S) is proposed to explain the effect of substrate on the oxidation of the flavoprotein. The kinetic fate of this complex is determined by the dissociation constant, $K_D^{E_RS}$, and the rate constant k_4''. Thus, the binding of substrate and the concomitant subtle changes in the enzyme structure mimic the effect of product binding via a specific modulation of the reaction with O_2 characterized by the rate constants k_4, k_4', and k_4''.

The experimental evidence for this expanded scheme is as follows. In previous studies of the kinetic mechanism, the occurrence of the ternary complex E_R–P–O_2 was postulated on the basis of direct evidence. In the present study the increased rate of reoxidation of the MPTP-reduced enzyme in the presence of added $MPDP^+$, the primary product, provides direct evidence for the participation of this species in the

Table III: Kinetic Parameters for Monoamine Oxidase from Steady-State Half-Reaction Experiments

	steady state		reductive half-reaction		oxidative half-reaction	
	turnover no. (s^{-1})	K_m (mM)	k_{obsd} (s^{-1})	K_D (mM)	app 2nd-order rate const (M^{-1} s^{-1})	app 1st-order rate const at air satn (s^{-1})
benzylamine	8.8	0.38	10.9	0.14	29400	7.6
MPTP	3.3	0.30	3.7	0.037	23300	6.0
MPDP	0.28	0.48	nd[a]	nd[a]	21800	5.6
dithionite[b]			nd[a]	nd[a]	6000	1.6

[a] nd indicates not determined. [b] From Husain et al. (1982).

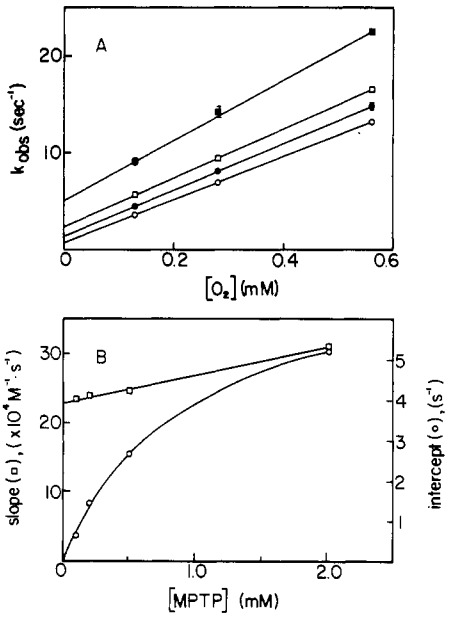


FIGURE 6: Dependence on oxygen concentration of observed rate constant for reoxidation of monoamine oxidase in the presence of excess MPTP. The data came from the same experiment as shown in Figure 5. (A) The final MPTP concentration was 0.1 (○), 0.2 (●), 0.5 (□), or 2 mM (■). (B) Secondary plot of the slopes (□) and intercepts (○) from (A).

reaction mechanism (Figure 2). Figures 3 and 5 show that increasing the concentration of either benzylamine or MPTP increased the observed rate of the oxidative half-reaction at all O_2 concentrations tested. This points to the existence of another ternary complex, E_R–S–O_2. As shown in Figures 4B and 6B, the slopes of the second-order rate plots for reoxidation of the reduced enzyme increased with benzylamine and MPTP concentrations, which implies that the formation of a ternary complex with substrate increases the rate of reoxidation. The best estimate of k_4'', i.e., the rate of reoxidation of the reduced enzyme in the E_R–S–O_2 complex, must be greater than the highest experimental value in Figure 4B (46×10^3 M^{-1} s^{-1}). Inspection of Table III shows that this rate is significantly higher than the second-order rate constant for the reoxidation of the free, fully reduced enzyme [0.6×10^4 M^{-1} s^{-1}, from Husain et al. (1982)] and also higher than the rate we observed in this study for the reoxidation of the semiquinone form of the free reduced enzyme (0.84×10^4 M^{-1} s^{-1}, data not shown). Also, since the rate of reoxidation of the reduced enzyme was exactly the same at all added $MPDP^+$ concentrations (see text and legend of Figure 2), the slightly higher value observed in the presence of excess MPTP may suggest that the two ternary complexes react at different rates.

As regards the dissociation constants of the E_R–S complexes, the data in Figures 4B and 6B show that second-order rate constants (slopes) increase in the experimental range. Therefore, the K_D values of E_R–S for MPTP and benzylamine must be large, probably >2 mM. The observation that this value is several orders of magnitude larger than the dissociation constant of substrate for oxidized enzyme represents no problem since the substrate binds to different forms of the enzyme.

The data shown in Figure 2 indicate that the rate of reoxidation of reduced enzyme is independent of the concentration of excess $MPDP^+$. The most likely interpretation is that the dissociation constant for E_R–$MPDP^+$ is very low (on the order of micromolar) and that the enzyme was saturated in the concentration range of $MPDP^+$ used. Since $MPDP^+$ is a slow substrate of the enzyme (Table III), one may be confident that the second-order rate constant in Table III (2.1×10^4 M^{-1} s^{-1}) reflects the rate of oxidation of E_R in the E_R–P–O_2 complex, without significant interference due to re-reduction of the oxidized enzyme produced. Thus the use of $MPDP^+$ represents an important opportunity to measure directly the effect of product on the oxidation of E_R.

As detailed under Results, Table III gives a comparison of the steady-state kinetic parameters with the rate constants for both half-reactions for benzylamine, MPTP, and $MPDP^+$. For benzylamine, the steady-state turnover number (8.8 s^{-1}) is numerically close to both the limiting rate for the reductive half-reaction and the apparent rate of reoxidation at an air saturated oxygen concentration of 258 μM. Consequently, for benzylamine the steady-state rate is determined by a complex function of both the reductive and oxidative half-reactions. For MPTP, the steady-state turnover is clearly dominated by the reductive half-reaction. The rate of the reductive half-reaction for $MPDP^+$ has not been determined; however, the reductive half-reaction must dominate the steady-state turnover because the oxidative half-reaction occurs at a rate nearly equal to that of MPTP.

An important further outcome of this work has been the development of a computer-based analytical procedure for the analysis of reaction sequences, such as those represented in eq 1. Since the procedure yields considerably more precise estimates of the parameters than the graphical method (Strickland et al., 1982) and since it appears to be generally applicable, it is detailed in the Appendix.

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APPENDIX

Considering eq 4, the analytical expression describing the double-reciprocal plot for the case $E + S \rightleftharpoons ES \rightarrow EP$, several points can be made. First, at the Y axis ($1/[S] = 0$) the value of $1/k_{obsd}$ is indeterminant. Consequently, the Y-axis intercept must be taken in the limit as $1/[S] \rightarrow 0$. The shape of the function for real nonnegative rates is concave upward, with an initial tangent at the Y axis of

$$1/k_{obsd} = (k_2/k_1k_3)(1/[S]) + 1/k_3 \qquad (10)$$

Consequently, the apparent Y-axis intercept is a good measure

of k_3. As $1/[S]$ gets large, eq 9 takes the following form in the asymptote:

$$1/k_{obsd} = [(k_2 + k_3)/k_1k_3](1/[S]) + k_2/[k_3(k_2 + k_3)] \quad (11)$$

Note that the Y-axis intercept of this asymptotic solution is nonzero for nonzero k_2. In the limiting case of $k_2 \gg k_3$, eq 10 and 11 become equal and the observed plot is linear. If $k_2 \ll k_3$, the scheme becomes one of consecutive first-order irreversible reactions and the relative amplitudes of the two exponentials depends on the relative values of $k_1[S]$ and k_3. Whichever rate is smaller will have the larger amplitude.

In general, double-reciprocal plots like Figure 1 have three measurable parameters: the Y-axis intercept (inter), the initial slope (inits), and the asymptotic slope (asymps). These can be combined to give the three rate constants as the initial point for a nonlinear least-squares analysis, viz.

$$k_1 = \frac{1}{(\text{asymps} - \text{inits})}$$

$$k_2 = \frac{1}{\text{inter}[(\text{asymps}/\text{inits}) - 1]}$$

$$k_3 = \frac{1}{\text{inter}}$$

Depending upon the degree of curvature in the double-reciprocal plot, the asymptotic slope may be severely underestimated if a simple graphical procedure is used. Conversely, if $k_2 \gg k_3$, the plot will appear to be linear and the asymptotic slope can be determined readily. In other words, the rapid equilibrium assumption will be valid. In cases of pronounced curvature, as seen in Figure 1, it seems that much better results are obtained by fitting all of the data at once rather than attempting to determine the parameters graphically.

References

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Chiba, K., Peterson, L. A., Castagnoli, K. P., Trevor, A. J., & Castagnoli, N., Jr. (1985) *Drug Metab. Dispos. 13*, 343–347.

Hiromi, K. (1979) *Kinetics of Fast Enzyme Reactions*, Halsted Press, New York.

Husain, M., Edmondson, D. E., & Singer, T. P. (1982) *Biochemistry 21*, 595–600.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.

Pearce, L. B., & Roth, J. A. (1985) *Biochemistry 24*, 1821–1826.

Ramsay, R. R., Husain, M., & Steenkamp, D. J. (1987) *Biochem. J. 241*, 883–892.

Salach, J. I. (1979) *Arch. Biochem. Biophys. 192*, 128–137.

Salach, J. I., Singer, T. P., Castagnoli, N., Jr., & Trevor, A. (1984) *Biochem. Biophys. Res. Commun. 125*, 831–835.

Singer, T. P., Salach, J. I., & Crabtree, D. (1985) *Biochem. Biophys. Res. Commun. 127*, 707–712.

Singer, T. P., Salach, J. I., Castagnoli, N., Jr., & Trevor, A. (1986a) *Biochem. J. 235*, 785–789.

Singer, T. P., Salach, J. I., Castagnoli, N., Jr., & Trevor, A. (1986b) in *MPTP: A Neurotoxin Producing a Parkinsonian Syndrome* (Markey, S. P., Castagnoli, N., Jr., Trevor, A., & Kopin, I. J., Eds.) pp 235–251, Academic, New York.

Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048–4052.

Mechanism and Requirements of in Vitro RNA Splicing of the Primary Transcript from the T4 Bacteriophage Thymidylate Synthase Gene†

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ABSTRACT: The splicing of a procaryotic precursor RNA transcribed from the T4 phage thymidylate synthase (*td*) gene with SP6 RNA polymerase was investigated in vitro. The intron excision–cyclization reaction increased progressively to 60 °C. Exon ligation, though barely detectable at the lower temperatures, was greatly enhanced at 60 °C. Both reactions required Mg^{2+}. The addition of guanosine to the 5′ end of an intron–exon II intermediate via a 3′,5′-phosphodiester bond was essential for the ligation of exon I to exon II. The added guanosine and the first intron-encoded uridine are subsequently lost as a dinucleotide from the 5′ end during cyclization of the linear form of the excised intron RNA. Exon ligation is intramolecular and occurs more readily in the nascent RNA molecule (cotranscriptionally) than in the finished transcript (posttranscriptionally). These data and the identification of various structural elements (P, Q, R, S, E, E′) in the *td* intron that are found typically in eucaryotic class I introns firmly establish the *td* intron as the first example of class I intron of procaryotic origin.

The presence of introns in eucaryotic genes has been amply documented. The expression of these genes involves RNA splicing reactions that are either small nuclear ribonucleoprotein particle (snRNP) dependent (Lerner et al., 1980; Mount et al., 1983; Greer et al., 1983; Peebles et al., 1983; Pikielny et al., 1983; Padgett et al., 1983; Kramer et al., 1984) or self-splicing (Cech et al., 1981; Kruger et al., 1983; Zaug et al., 1983; Tabak et al., 1984; Garriga & Lambowitz, 1984). Examples of self-splicing RNAs include the precursors of *Tetrahymena thermophila* nuclear rRNA, yeast and fungal mitochondrial rRNA and mRNA, and some chloroplast

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