Kinetic Studies on the Catalytic Mechanism of Liver Monoamine Oxidase†

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ABSTRACT: The kinetic mechanism of mitochondrial monoamine oxidase from beef liver has been investigated by steady-state and pre-steady-state techniques. Parallel line double-reciprocal plots were observed with all substrates tested by using either conventional or stopped-flow-monitored steady-state approaches. A maximal velocity of ~800 min^{-1} at infinite concentrations of benzylamine and oxygen was observed at 25 °C with either technique, showing that the catalytic activity is not affected by a 10^3-fold change in enzyme concentration. Steady-state studies comparing [α,α-$^2H_2$]-benzylamine and benzylamine gave a kinetic isotope effect of 6.4–6.7, with no effect on K_m values for benzylamine and O_2. The rate of reduction of monoamine oxidase determined anaerobically in stopped-flow experiments gave an extrapolated value of k_3 = 700 min^{-1} and a k_H/k_D of 8.7. No isotope effect was observed on substrate binding (K_S). The rate of reoxidation of substrate- or dithionite-reduced monoamine oxidase by molecular oxygen followed second-order kinetics (k = 3.6 × 10^5 M^{-1} min^{-1}). Analysis of these kinetic data shows that reduction of the enzyme by benzylamine is rate limiting in catalysis and that oxygen must react with the reduced enzyme–product complex, rather than with the free reduced enzyme. A similar approach using β-phenylethylamine as substrate showed virtually no kinetic isotope effect in steady-state catalytic assays. The rate of reduction of the enzyme (k_3 = 34 300 min^{-1}, k_H/k_D = 3) was much faster than the catalytic turnover (1250 min^{-1}). These results in conjunction with those of the oxidative half-reaction strongly suggest that the rate-limiting step in the oxidation of β-phenylethylamine is the reaction of the free, reduced enzyme with oxygen. It may be concluded from these data that, depending on the nature of the substrate, the kinetic mechanism followed by monoamine oxidase may involve either a ternary or a binary complex (ping-pong) mechanism.

Interest in monoamine oxidase has increased markedly in recent years. One reason for this may be that the elucidation of several features of the catalytic site of monoamine oxidase, including the structure of the covalently bound flavin and the sequence of amino acids attached to it (Kearney et al., 1971; Walker et al., 1971), has led to clarification of the mechanism of the suicide inactivation of the enzyme by most pharmacologically and clinically important compounds of which it is the target enzyme (Singer, 1979; Singer & Salach, 1981).

Although nearly homogeneous preparations of the B form of monoamine oxidase from beef liver and kidney have been available for several years (Chuang et al., 1974; Minamiura & Yasunobu, 1978; Salach, 1979), and recently a procedure for the isolation of the pure A enzyme has been reported (Zeller, 1981), the catalytic mechanism of this interesting and important enzyme has not been elucidated. Published reports are restricted to steady-state kinetic analyses of the B form of the enzyme. Using a variety of substrates and sources of the enzyme, it has been reported that double-reciprocal plots yield parallel lines, and from this, it was generally concluded that the enzyme acts by a ping-pong mechanism (Tipton, 1968; Oi et al., 1970, 1971; Fowler & Oreland, 1979; Roth, 1979). Studies on D-amino acid oxidase (Palmer & Massey, 1968; Bright & Porter, 1975) have demonstrated, however, that such a conclusion may only be made safely, at least with flavoprotein oxidases, if steady-state kinetic data are supported by pre-steady-state data. Such studies have recently become possible by virtue of improvements in preparative procedures for the B enzyme (Salach, 1979; Weyler & Salach, 1981) which permit isolation of substantial quantities of the protein in homogeneous form and free from iron-containing impurities.

This manuscript describes steady-state, pre-steady-state, and stopped-flow turnover studies on monoamine oxidase. The approach is patterned on the kinetic procedures outlined by Bright & Porter (1975) for flavoenzyme oxidases. The data presented below show that the mechanism of monoamine oxidase is a function of the nature of the substrate.

Materials and Methods

Enzymes and Reagents. Monoamine oxidase was isolated from bovine liver mitochondria according to the method published by Salach (1979) or Weyler & Salach (1981). Active site concentrations were determined by [^{14}C]pargyline binding (Salach, 1979). Benzylamine, β-phenylethylamine hydrochloride, and crystalline catalase were from Sigma Chemical Co., and reagent-grade glucose oxidase was from Miles Laboratories, Inc. Benzylamine was recrystallized from ethanol as its hydrochloride. β-[α,α-$^2H_2$]Phenylethylamine (Merck, Sharp & Dohme, Canada; isotopic purity = 98%) was converted to the hydrochloride by adding concentrated HCl to an ethereal solution of the amine.

[α,α-$^2H_2$]Benzylamine was synthesized by reducing redistilled benzonitrile (Aldrich) with lithium aluminum [^{2}H]-hydride (Stohler Isotope Chemical Co.) in anhydrous ethyl ether, according to the procedure of Amundsen & Nelson (1951). After recrystallization of the product, the isotopic purity was monitored by ^{1}H NMR spectroscopy. No detectable α-methylene protons were observed. [^{14}C]Pargyline (1.12 mCi/mmol) was a gift of Dr. R. C. Sanders, Abbot Laboratories.

Kinetic Studies. Unless otherwise stated, kinetic experiments were performed at 25 °C in 50 mM *N*-(2-hydroxy-

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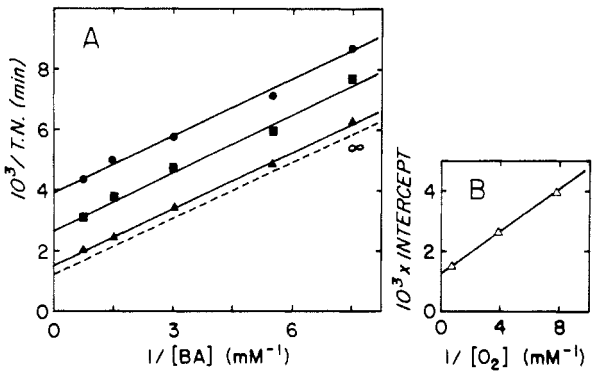


FIGURE 1: (A) Double-reciprocal plot of initial data for the oxidation of benzylamine by beef liver mitochondrial monoamine oxidase. Assays were performed in 50 mM Hepes and 0.5% Triton X-100, pH 7.5, 25 °C, as a function of benzylamine concentration at the following fixed concentrations of oxygen as described under Materials and Methods: (●) 0.13; (■) 0.26; and (▲) 1.3 mM. The initial rates measured by following the increase in absorbance at 250 nm (Tabor et al., 1960) are plotted as mol min^{-1} (mol of enzyme)$^{-1}$. (B) Secondary plot of intercepts from the data in (A) against the reciprocals of the oxygen concentration. BA denotes benzylamine.

ethyl)piperazine-*N*'-2-ethanesulfonic acid (Hepes[1]) buffer, pH 7.5, containing 0.5% (w/v) Triton X-100. The presence of this detergent was necessary to maintain the clarity of the enzyme solutions during spectral studies.

Initial rates of oxidation of benzylamine (both α,α-^{1}H and α,α-^{2}H) were measured spectrophotometrically in a total volume of 3 mL by following the increase in absorbance at 250 nm due to benzaldehyde formation (Tabor et al., 1960). When initial rates were measured at O_2 concentrations other than at air saturation, the assay cuvettes were fitted with silicone rubber stoppers and flushed with a gas mixture containing the appropriate oxygen concentration. Initial rate studies using β-phenylethylamine as substrate were performed with an oxygen electrode, using a total volume of 1.2 mL. The sensitivity of the instrument permitted the measurement of oxygen concentration in the range of 10 μM with good precision.

Rapid reaction kinetic experiments were performed with an Aminco-Morrow stopped-flow apparatus interfaced with a Nova 2/4 minicomputer, using the hardware and software supplied by On-Line Instrument Systems. Stopped-flow enzyme-monitored turnover experiments were carried out as described by Gibson et al. (1964) [cf. also Bright & Porter (1975) for a good description of this technique as applied to flavoenzymes]. The rate of reduction of monoamine oxidase by various substrates was monitored in the stopped-flow apparatus under anaerobic conditions. Enzyme and substrate solutions in glass tonometers were made anaerobic by evacuation and flushing 8 times with argon which had been passed over Cu filings at 450 °C to remove residual O_2 and subsequently water saturated. The drive syringes and mixing chamber of the stopped-flow apparatus were scrubbed of oxygen by flushing with a solution of reduced riboflavin, followed by degassed buffer. The chamber of the apparatus housing the mixing chamber, the observation cell, and the tubes leading from the drive syringes were under a positive pressure of argon in order to maintain anaerobiosis during the kinetic experiments. The rate of reaction of reduced monoamine oxidase with oxygen was also measured in the stopped-flow apparatus. Anaerobic solutions of the enzyme containing 10 μM glucose oxidase, 55 nM catalase, and 10 mM glucose were reduced by the addition of stoichiometric quantities of benzylamine or dithionite. The rates of oxidation were followed on mixing with an equal volume of buffer containing various concentrations of oxygen. All kinetic data reported in this paper are presented as turnover numbers (moles of substrate utilized per minute per mole of enzyme–flavin).

[1] Abbreviation: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

Results

Steady-State Kinetics. In order to permit direct comparison with the results of stopped-flow experiments, the rates of oxidation of benzylamine, β-phenylethylamine, and of their deuterated analogues were determined by conventional steady-state methods. Figure 1A is a double-reciprocal plot of the rates of benzylamine oxidation at varying O_2 concentrations. In agreement with previous data for the enzyme from beef liver (Oi et al., 1970), a parallel line pattern was obtained. Comparable results were found when [α,α-$^2H_2$]benzylamine, β-phenylethylamine, and β-[α,α-$^2H_2$]phenylethylamine served as substrates. These data are consistent with the following steady-state rate equation (Dalziel, 1957):

$$\frac{E_t}{V_0} = \phi_0 + \frac{\phi_S}{[S]} + \frac{\phi_{O_2}}{[O_2]} \quad (1)$$

where E_t is the total concentration of catalytic centers, V_0 is the initial rate, ϕ_0 is the reciprocal of the maximum turnover number, and ϕ_S and ϕ_{O_2} are the apparent bimolecular constants involved in the two half-reactions. The various kinetic parameters shown in this equation have been calculated from experiments, such as that in Figure 1, and are summarized in Table I.

Table II compares the Michaelis constants for the substrate and for O_2 and the V_{max} values for benzylamine, β-phenylethylamine, and their dideuterated forms. It may be noted that in the case of benzylamine the K_m values for the substrate and for O_2 do not change significantly; when deuterium is introduced in the α position, the V_{max} value shows a 6.7-fold kinetic isotope effect, showing that in the oxidation of this substrate proton removal, i.e., the reductive half-reaction, is rate limiting. In contrast, with β-phenylethylamine, deuterium incorporation has only a trivial effect on the maximal velocity, suggesting that in that case the reductive step is not rate limiting. The K_m for O_2 is 10 times higher with this substrate than with benzylamine (Table II). An interesting consequence is that while in air the maximum turnover rate for benzylamine (400 min^{-1} at 25 °C) is 3.3 times higher than that for β-phenylethylamine (120 min^{-1}); at infinite O_2 concentration, the maximal rate of oxidation of benzylamine is about 60% of that of β-phenylethylamine.

Stopped-Flow-Monitored Turnover Experiments. It has been well documented for various flavoprotein oxidases that the measurement of the extent of reduction of the flavin as a function of time during steady-state catalytic turnover provides valuable mechanistic information which can be correlated with the results of steady-state kinetic experiments (Gibson et al., 1964; Bright & Porter, 1975). Flavoenzymes are particularly amenable to this approach, because the absorption spectrum of the flavin chromophore in the visible region is usually distinct from those of the substrate and products.

Figure 2 shows the time course of absorbance changes at 450 nm at three benzylamine concentrations (1, 2, and 10 mM) and 0.26 mM initial O_2 concentration. In each experiment, there was a rapid, initial bleaching of the flavin absorbance, followed by slower changes, until the enzyme-bound

Table I: Experimental Values of Various Steady-State Kinetic Coefficients for the Oxidation of Different Amines by Beef Liver Mitochondrial Monoamine Oxidase

kinetic coefficient	kinetic equivalent [a]	substrates tested: benzylamine	[α,α-$^2H_2$]-benzylamine	β-phenylethylamine	β-[α,α-$^2H_2$]phenylethylamine
ϕ_0 (min)	$1/k_3 + 1/k_5$	1.25×10^{-3} 1.35×10^{-3} [b]	8.3×10^{-3} 8.7×10^{-3} [b]	8×10^{-4}	8.75×10^{-4}
ϕ_S (M min)	$k_2/(k_1k_3)$	6.35×10^{-7} 7.8×10^{-7} [b]	4×10^{-6}	1.5×10^{-7}	2×10^{-7}
ϕ_{O_2} (M min)	$1/k_4$	3.5×10^{-7} 3.6×10^{-7} [b]	1.47×10^{-6} 1.8×10^{-6} [b]	2.3×10^{-6}	2.2×10^{-6}
$\phi_{O_2}^{-1}$ (M^{-1} min^{-1})	k_4	2.8×10^6	6.1×10^5	4.3×10^5	4.5×10^5

[a] Derived for the mechanism illustrated in Scheme I. [b] Calculated from stopped-flow-monitored turnover data.

Table II: Steady-State Kinetic Parameters of Monoamine Oxidase with Various Substrates

kinetic parameter	benzylamine	[α,α-$^2H_2$]-benzylamine	β-phenylethylamine	β-[α,α-$^2H_2$]-phenylethylamine
K_m^S (mM) [a]	0.51 0.58 [b]	0.48 0.46 [b]	0.19	0.25
$K_m^{O_2}$ (mM) [a]	0.28 0.27 [b]	0.18 0.21 [b]	2.8	2.6
V_{max} (min^{-1}) [c]	800 740 [b]	120 115 [b]	1250	1140

[a] The Michaelis constant for each substrate is the concentration of the substrate that gives half the true maximum velocity in the presence of infinitely large concentrations of the other substrate. It is calculated by dividing ϕ_S by ϕ_0 (Dalziel, 1957). [b] Calculated from stopped-flow-monitored turnover data. [c] Maximal velocity of the reaction at infinite concentrations of both amine and oxygen and was calculated from the relationship $V_{max} = 1/\phi_0$ (Dalziel, 1957).

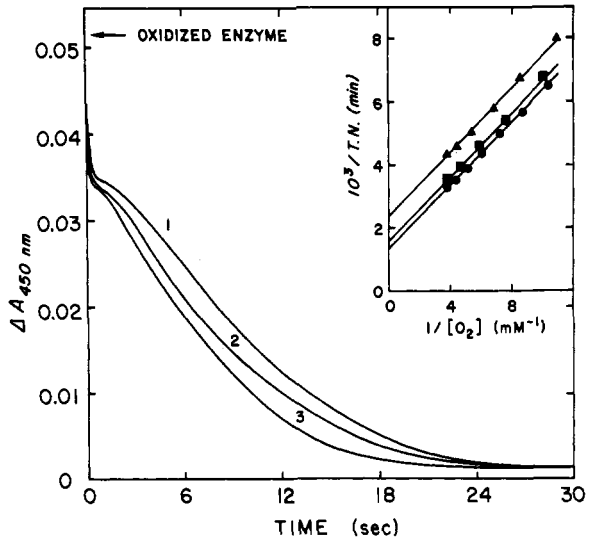


FIGURE 2: Stopped-flow-monitored turnover experiments at the following final concentrations of benzylamine: (1) 1; (2) 2; and (3) 10 mM. Monoamine oxidase at a final concentration of 6.8 μM was mixed with different concentrations of benzylamine, and the absorbance changes at 450 nm were recorded with time. The reaction was carried out at 25 °C in 50 mM Hepes and 0.5% Triton X-100, pH 7.5, at an initial concentration of oxygen of 0.26 mM. The data were analyzed by the method of Gibson et al. (1964).

flavin became fully reduced owing to the exhaustion of O_2. When the data are analyzed by the method of Gibson et al. (1964), a set of parallel lines are obtained (Figure 2, inset). Repetition of this experiment at wavelengths other than 450 nm disclosed no evidence for the occurrence of any spectral intermediate forms during turnover. The kinetic constants obtained from stopped-flow-monitored turnover experiments are listed in Tables I and II. These constants are in good agreement with the values obtained by conventional steady-state experiments. This rules out any anomalous kinetic behavior caused by an increase of $\sim 10^3$ in enzyme concentration.

The conclusion from steady-date data that the rate-limiting step in the action of monoamine oxidase varies with the substrate was confirmed by turnover experiments. Figure 3 compares the time course of absorbance changes at 450 nm for benzylamine, dideuteriobenzylamine, β-phenylethylamine, and kynuramine. The extent of reduction of the flavin during the steady-state phase is an indication of the relative rates of reduction of the enzyme by the particular substrate and of its reoxidation by O_2. In the case of benzylamine, about 40% of the enzyme is in the reduced state on entering the steady state, while with [α,α-$^2H_2$]benzylamine only 5% is reduced. This is consistent with the very large kinetic isotope effect noted in steady-state kinetic assays. By contrast, during the oxidation of β-phenylethylamine, some 80–90% of the enzyme is in the reduced form when entering the steady state, confirming the finding that with this substrate the reductive half-reaction is far faster than the oxidative one. The same appears to be true for kynuramine (Figure 3). Because of the low level of oxidized enzyme found in the steady state with these two substrates and its further subsequent decrease on depletion of oxygen, the data were not analyzed to determine the relevant kinetic constants because of the large inherent errors involved.

Reductive Half-Reaction. Anaerobic stopped-flow experiments in which the oxidized enzyme was mixed with excess substrate showed rapid reduction to the flavin hydroquinone. Analysis of the kinetic traces at various wavelengths gave no indication of the formation of a flavoenzyme radical during the reaction. The reduction of monoamine oxidase followed pseudo-first-order kinetics and depended on the substrate concentration. Figure 4 is a double-reciprocal plot of the rate constants of the reductive half-reaction vs. benzylamine or [α,α-$^2H_2$]benzylamine concentration. The data confirm the large kinetic isotope effect and show saturation kinetics for both substrates. Similar linear plots were obtained for β-phenylethylamine and β-[α,α-$^2H_2$]phenylethylamine (data not shown). These data demonstrate that a rapid equilibrium exists between the enzyme and substrate, followed by a slower rate of reduction of the enzyme-bound flavin:

$$E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\text{–}S \xrightarrow{k_3} E_R P \qquad (2)$$

where $k_3 \ll k_2$, $k_1[S]$.

The dissociation constants for the enzyme–substrate complex (k_2/k_1), as well as the limiting values for the rate of reduction (k_3), may be calculated from the slopes and intercepts of double-recirocal plots such as those in Figure 4 (Strickland et al., 1975). The slope yields directly the value for the ϕ_S coefficient of eq 1 (Bright & Porter, 1975). Values for the

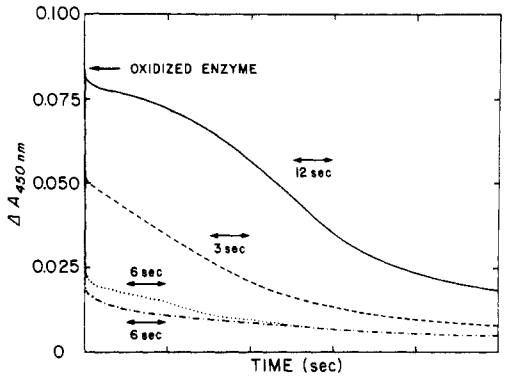


FIGURE 3: Variation in the steady-state concentration of the oxidized enzyme during stopped-flow-monitored turnover experiments in the presence of different substrates. The enzyme (final concentration ~7 μM) was mixed in the stopped-flow spectrophotometer with equal volumes of solutions containing excess of substrates and the decrease in absorbance at 450 nm recorded for the time indicated. The following substrates were used: (—) 10 mM [α,α-$^2H_2$]benzylamine; (- - -) 10 mM benzylamine; (···) 10 mM kynuramine; (-·-·-) 20 mM β-phenylethylamine. The reaction conditions were identical with those in the legend of Figure 2.

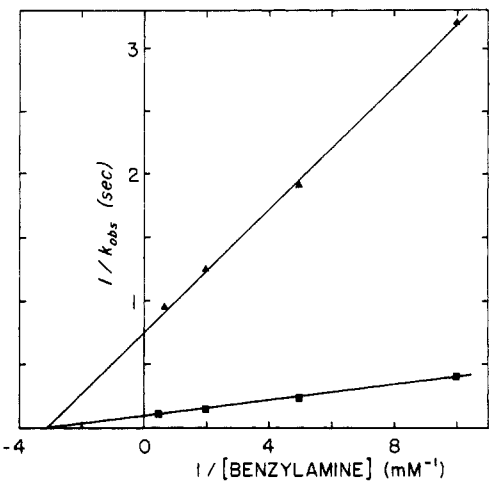


FIGURE 4: Deuterium isotope effect on the apparent first-order rate constant for the reduction of monoamine oxidase by benzylamine. The reactions were carried out anaerobically in 50 mM Hepes and 0.5% Triton X-100, pH 7.5, at 25 °C, by mixing in the stopped-flow spectrophotometer equal volumes of monoamine oxidase (final concentration ~7 μM) and solutions containing different amounts of benzylamine (■) or [α,α-$^2H_2$]benzylamine (▲). The apparent first-order rate constants calculated from the absorbance changes at 450 nm for the reduction of the enzyme are plotted in the double-reciprocal manner.

dissociation constants, k_3, and ϕ_S for the various substrates tested are listed in Table III. The agreement between the values of k_3 and ϕ_0^{-1} (from steady-state data), as well as the kinetic isotope effect for benzylamine and [α,α-$^2H_2$]benzylamine, confirms the conclusion that, in the oxidation of this substrate, reduction of the enzyme is the rate-determining step in catalysis. Further, the agreement observed between the ϕ_S coefficient calculated from steady-state data (Table I) and from reductive half-reaction data (Table III) provides strong evidence that eq 2 is a correct representation of the reductive half-reaction. It should also be noted that such a scheme is a sufficient condition to give the parallel line patterns observed in the double-reciprocal plots of steady-state data.

The value of k_3 observed for the reduction of monoamine oxidase by β-phenylethylamine is approximately 30-fold larger than the corresponding turnover number determined from steady-state data. Thus, in the case of this substrate, in contrast to benzylamine, the rate-limiting step in catalysis must be a step other than reduction of the enzyme.

Oxidative Half-Reaction. The rate of reaction of the reduced enzyme with O_2 was determined spectrophotometrically in the stopped-flow apparatus by following the rate of appearance of the absorbance at 450 nm. The reduced enzyme was prepared by the anaerobic addition of a slight excess of benzylamine or of dithionite. Figure 5 is a plot of the apparent first-order rate constants as a function of O_2 concentration. The linear plot, passing through the origin, is consistent with a second-order reaction, with no indication of the formation of a reduced enzyme–O_2 complex. If such a complex is formed, it must decay to oxidized enzyme and H_2O_2 faster than it is being formed.

The second-order rate constant obtained was the same (3.6 × 10^5 M^{-1} min^{-1}) whether the enzyme was reduced with benzylamine or with dithionite. At air saturation, the value of the apparent first-order rate constant for oxidation of reduced enzyme (93.6 min^{-1}) was considerably less than the apparent turnover number with benzylamine (400 min^{-1}). These data suggest that during the oxidation of benzylamine O_2 must react with the reduced enzyme–product complex, rather than with the free reduced enzyme, and that this reaction is considerably faster than the oxidation of the free reduced enzyme.

Attempts were made to form a reduced enzyme–imine complex by incubating reduced monoamine oxidase for several minutes with a 10-fold excess of benzaldehyde and a large excess of NH_4^+ (~0.1 M). A biphasic oxidation of the enzyme-bound flavin was observed on mixing with O_2. About 25% of the total absorbance change showed an apparent rate constant similar to that of the free reduced enzyme with O_2, while ~75% was 4-fold slower. Although it is difficult to interpret these results, it should be noted that Oi et al. (1970) have published kinetic evidence for the inhibition of the beef liver enzyme by low concentrations of benzaldehyde. Whether this is due to chemical reaction with thiol or ε-amino groups in the protein is not known.

While the kinetic data do not support a binary complex mechanism for monoamine oxidase when benzylamine is used as the substrate, the data do support the kinetic competence of the free reduced enzyme when β-phenylethylamine is the substrate. The second-order rate constant for O_2 oxidation of the reduced enzyme (3.6 × 10^5 M^{-1} min^{-1}) is comparable to $\phi_{O_2}^{-1}$ of 4.3 × 10^5 M^{-1} min^{-1} observed for β-phenylethylamine (Table I). Furthermore, the apparent turnover numbers (50 min^{-1} at 10% O_2 and 118 min^{-1} at 20% O_2) are close to the apparent first-order rate constants for oxidation of the

Table III: Kinetic Constants Calculated from the Reductive Half-Reaction Data

constant	values			
	benzylamine	[α,α-$^2H_2$]-benzylamine	β-phenylethylamine	β-[α,α-$^2H_2$]-phenylethylamine
k_3 (min^{-1}) [a]	700	80	34300	11290
K_S (k_2/k_1) (mM) [b]	0.36	0.33	4.5	3.3
ϕ_S (M min) [c]	5.2 × 10^{-7}	4 × 10^{-6}	1.2 × 10^{-7}	2.1 × 10^{-7}

[a] Calculated from the intercept values of the double-reciprocal plot such as shown in Figure 1 (Strickland et al., 1975). [b] The dissociation constant for the enzyme–substrate complex is calculated from the slope and intercept of the double-reciprocal plots (K_S = slope/intercept) (Strickland et al., 1975). [c] Calculated from the slope of the double-reciprocal plot (Bright & Porter, 1975).

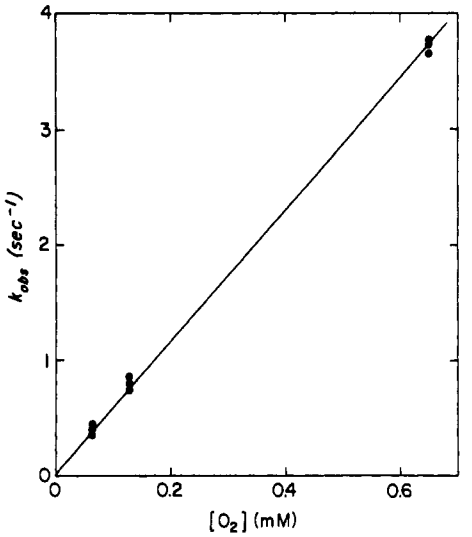


FIGURE 5: Variation with oxygen concentration of the apparent first-order rate constant for the reaction of the reduced enzyme with oxygen. The reaction was performed in the stopped-flow spectrophotometer by mixing anaerobic solutions of reduced monoamine oxidase (final concentration ~7 μM), prepared by addition of a slight excess of benzylamine, with an equal volume of the buffer solutions, which had been saturated with different concentrations of oxygen. The reoxidation of the reduced enzyme was monitored by following the increase in absorbance at 450 nm. The reaction conditions were identical with those of Figure 4.

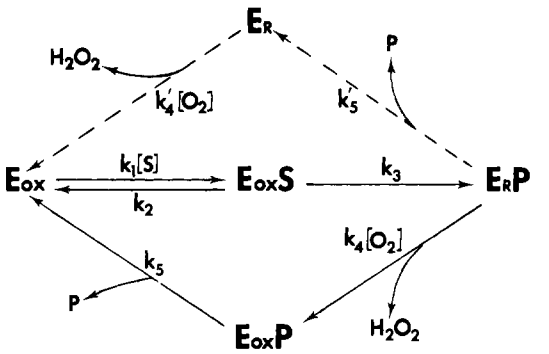
reduced enzyme observed at these O_2 concentrations (47 and 94 min^{-1}, respectively). In view of the observation that the rate of reduction of the enzyme by β-phenylethylamine proceeds 30-times faster than the maximal velocity of catalytic turnover, these results provide substantial evidence that for this substrate the reaction of the free, reduced enzyme with O_2 is the rate-limiting step in catalysis.

Discussion

Our purposes in undertaking this study were to determine if the catalytic mechanism of the B form of monoamine oxidase operates via a binary complex, a ternary complex, or both and to describe the kinetic constants for selected substrates which could serve as a basis for more intensive studies of the molecular basis of the catalytic activity of the enzyme in the future. For the comparison of these two approaches to be valid, it is essential to rule out kinetic anomalies caused by the large difference in enzyme concentrations used in steady-state and stopped-flow experiments, respectively. The observed agreement of steady-state kinetic parameters over a 10^3 difference in enzyme concentration (Tables I and II) is gratifying and rules out any interference in kinetic behavior due to enzyme aggregation and/or micelle formation.

The major kinetic isotope effect observed in steady-state assays for benzylamine and supporting data from stopped-flow experiments clearly show that the reductive half-reaction step is rate limiting. Since in these experiments both α hydrogens of benzylamine were replaced by deuterium, the observed effect represents a combination of both primary and secondary effects, of which the former predominates. It remains for future work to establish whether the *R* or *S* α hydrogen of benzylamine is involved in electron transfer to the flavin. Past studies using stereospecifically deuterated tyramine (Belleau et al., 1960) have shown the *S* hydrogen to be involved. We feel, however, that the extension of these results to benzylamine is not warranted, since a detailed kinetic analysis with tyramine to determine whether the reductive half-reaction is rate limiting has not yet been performed.

Scheme I



The demonstration that the turnover rate of the enzyme using benzylamine is much higher than the rate of reoxidation of the substrate- or dithionite-reduced enzyme strongly suggests that with this substrate a ternary complex mechanism operates (lower loop in Scheme I), rather than the binary complex mechanism suggested by Oi et al. (1970). In contrast, the data on β-phenylethylamine are most consistent with the view that, with this substrate, the binary complex mechanism (top loop of Scheme I) is followed and that the oxidation of the free, reduced enzyme is the rate-limiting step in catalysis. This notion is supported by the rapid rate of reduction of the enzyme, which is approximately 30-times that of the maximal velocity, and by the correlation with the observed rates of oxidation of the free, reduced enzyme at the same O_2 concentrations. Consistent with this latter observation are the similar values for $\phi_{O_2}^{-1}$ of 4.3×10^5 M^{-1} min^{-1} for β-phenylethylamine and the observed second-order rate constant of 3.6×10^5 M^{-1} min^{-1} for the oxidation of reduced enzyme by O_2. Moreover, although a kinetic isotope effect in the reductive half-reaction is evident in comparing β-phenylethylamine and its dideuterio analogue (Table III), as expected from the fact that with this substrate the oxidative step is rate limiting, steady-state data show almost no kinetic isotope effect (Table II).

These data show that, in common with other flavoprotein oxidases (except glucose oxidase, which appears to function exclusively by a binary complex mechanism) (Bright & Porter, 1975), monoamine oxidase catalysis can follow either the upper or the lower loop of Scheme I, depending on the nature of the substrate. The choice of pathway is probably dictated by the relative magnitude of $k_4[O_2]$ vs. k'_5. If k'_5 is greater than $k_4[O_2]$, then the top loop will be followed, as is the case with β-phenylethylamine. Conversely, with benzylamine, $k_4[O_2]$ is greater than k'_5, and consequently, the bottom loop is followed. The structural nature of the intermediate imine and its interaction with the enzyme-bound flavin, as well as other groups about the catalytic site, thus determine the mechanistic path. It is conceivable that with certain substrates both paths might be followed when $k_4[O_2] \approx k'_5$, which would result in the appearance of curved but parallel double-reciprocal plots, as found for the oxidation of L-phenylalanine by L-amino acid oxidase (Porter, 1972).

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