Spectral and Kinetic Studies of Imine Product Formation in the Oxidation of *p*-(*N*,*N*-Dimethylamino)benzylamine Analogues by Monoamine Oxidase B†

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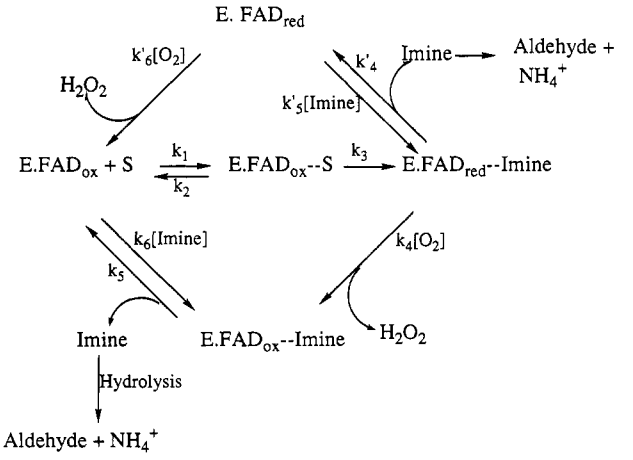
ABSTRACT: The oxidative deamination of *p*-(*N*,*N*-dimethylamino)benzylamine and *N*-methyl-*p*-(*N*,*N*-dimethylamino)benzylamine by bovine liver monoamine oxidase B has been investigated by absorption spectral, steady-state, and stopped-flow kinetic studies. An absorbing intermediate with a maximum at 390 nm is observed with either analogue in turnover experiments at neutral pH and is identified as due to the formation of protonated imine as the initial product. *p*-(*N*,*N*-Dimethylamino)benzaldehyde is the final product formed from either substrate analogue. Anaerobic stopped-flow measurements show *N*-methyl-*p*-(*N*,*N*-dimethylamino)benzylamine to reduce enzyme-bound flavin with a limiting rate of 1.8 s^{-1} concurrent with the appearance of a 390-nm absorption due to protonated imine product with a limiting rate of 1.7 s^{-1}. Both observed rates are somewhat faster than catalytic turnover (1.5 s^{-1}). Under anaerobic conditions, the decay of protonated *N*-methyl-*p*-(*N*,*N*-dimethylamino)benzenimine is much slower than turnover ($k = 4.8 \times 10^4$ s^{-1}). *p*-(*N*,*N*-Dimethylamino)benzylamine reduces the enzyme with a limiting rate of 2.1 s^{-1}, which is faster than catalytic turnover (1.2 s^{-1}). Protonated imine formation is also observed with this substrate with an apparent limiting rate of 1.3 s^{-1}. The decay of the protonated *p*-(*N*,*N*-dimethylamino)benzenimine absorbance is slower than catalytic turnover but faster than the rate of aldehyde formation under anaerobic conditions. Deuterium kinetic isotope effect values of ~10 are observed both for flavin reduction and for protonated imine formation. No isotope effect is observed for the rate of imine decay. These results demonstrate that the protonated form of the imine is the initial product in the oxidation of benzylamines by monoamine oxidase B. The reduced enzyme–imine complex is kinetically stable to dissociation, but reacts with O_2 at rates consistent with catalysis. Imine hydrolysis occurs nonenzymatically after release from the oxidized form of the enzyme.

Monoamine oxidase (EC 1.4.3.4, MAO)[1] is a membrane-bound flavoprotein that catalyzes the oxidative deamination of biogenic amines using O_2 as the electron acceptor. Both the A and the B isozymic forms of the enzyme are situated on the mitochondrial outer membrane and exhibit differing substrate specificities. The B form of the enzyme can be purified in good yield from bovine liver mitochondria and has been the subject of a number of mechanistic studies.

Previous steady-state and stopped-flow kinetic studies on the interaction of purified MAO B with benzylamine and with phenethylamine (Husain et al., 1982) suggested that the nature of the substrate dictates the catalytic pathway for oxidative deamination. Benzylamine oxidation proceeds via a ternary complex mechanism wherein O_2 reacts with the reduced-enzyme–imine complex (bottom loop of Scheme I) to form H_2O_2 and the oxidized enzyme product complex. Phenethylamine oxidation proceeds by an alternate pathway, wherein the imine product readily dissociates from the reduced enzyme and O_2 subsequently reoxidizes the free, reduced enzyme to form H_2O_2 and the oxidized form of the enzyme (top loop of Scheme I).

Scheme I



This kinetic pathway (Scheme I) was subsequently shown to also occur for the native, membrane-bound form of MAO B from human brain (Pearce & Roth, 1985). Using steady-state inhibition data with D-amphetamine, Pearce and Roth (1985) showed that tyramine and tryptamine, like phenethylamine, are oxidatively deaminated by MAO B via a binary complex mechanism. A common structural element for these three substrates include an aromatic ring in the β-position to the primary aliphatic amine. The benzylamine data suggest that aromatic ring placement α to the aliphatic amine would in some way slow the rate of imine dissociation from the reduced enzyme and may, in fact, increase the rate of O_2

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[1] Abbreviations: MAO, monoamine oxidase; DMABA, *p*-(*N*,*N*-dimethylamino)benzylamine; DMABI, *p*-(*N*,*N*-dimethylamino)benzenimine; NMDMABA, *N*-methyl-*p*-(*N*,*N*-dimethylamino)benzylamine; NMDMABI, *N*-methyl-*p*-(*N*,*N*-dimethylamino)benzenimine; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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reactivity with the reduced flavin relative to that of the uncomplexed form of the reduced enzyme. Husain et al. (1982) showed that the $\phi(O_2)$ value (Dalziel, 1957) for phenethylamine oxidation was identical with the observed second-order rate constant for O_2 reaction with the reduced enzyme and that this value is 6–7-fold slower than the $\phi(O_2)$ value calculated when benzylamine is the substrate.

Other than kinetic arguments, no direct detection of a reduced-enzyme–product complex has been observed as a reaction pathway intermediate for MAO B catalysis. Stopped-flow absorbance studies in the visible region show only the reduction of the enzyme-bound covalent flavin from its oxidized to its hydroquinone form on reaction with benzylamine, with no detectable intermediates. In studies of benzylamine oxidation by soluble preparations from rat liver, Houslay and Tipton (1975) have suggested that the aldehyde product is released from the reduced enzyme but that NH_4^+ is released only subsequent to O_2 binding. These studies would imply that imine hydrolysis occurs on the reduced enzyme. Tipton et al. (1987) have suggested the presence of irreversible steps in product release upon imine hydrolysis to account for the kinetic differences observed on comparison of MAO B-catalyzed benzylamine oxidation with phenethylamine oxidation. The conversion of benzylamine to benzenimine might be observable in the ultraviolet region; however, absorption and light scattering due to the protein and to the detergent (Triton X-100) required to solubilize the enzyme interfere with and thus preclude this measurement.

Chromophoric substrate analogues have been used profitably to investigate the mechanisms of a number of enzyme systems (Bernhard et al., 1965; Hinkle & Kirsch, 1970; Malhatra & Bernhard, 1968; Dunn & Hutchinson, 1973) and to detect any spectral intermediates that may occur. The *p*-(*N*,*N*-dimethylamino)phenyl group has been valuable as a chromophoric substrate analogue to investigate alcohol dehydrogenase–substrate interactions by both rapid reaction and spectral techniques (Dunn & Hutchinson, 1973; Callendar et al., 1988; Dunn et al., 1975; Anglis et al., 1977; Jagodzinski et al., 1977). In the case of MAO B-catalyzed benzylamine oxidation, the placement of a *p*-(*N*,*N*-dimethylamino) group on the benzylamine ring would facilitate detection of the aldehyde product since it absorbs at 352 nm ($\epsilon = 3 \times 10^4\ M^{-1}\ cm^{-1}$). The imine, if detectable, might be expected to absorb at an even higher wavelength since the C–N bonds of the para substituent are shorter (more sp^2 character), which would favor the quinone tautomeric form on comparison of the X-ray structure of the oxime with that of the aldehyde (Bachecki & Zambonelli, 1973). Previous studies have shown that DMABA is a reasonable substrate for MAO B (Dietrich & Erwin, 1969; Tabakoff & Alivasatos, 1972; Hellerman & Erwin, 1968). The para substituent does not drastically influence substrate binding since *p*-(*N*,*N*-dimethylamino)-α,α-dimethylphenethylamine (FLA 336 in its (–) configuration) has been shown to be a potent competitive inhibitor of MAO B (Fowler & Oreland, 1981). As will be demonstrated in this article, MAO B catalyzes the formation of DMABI from DMABA and NMDMABI from NMDMABA with subsequent formation of the para-substituted benzaldehyde. These events can be observed spectrally, and they demonstrate the formation of the reduced-enzyme–product imine complex which, by virtue of its kinetic stability, is probably the reactive species with O_2 as predicted by previous kinetic data for benzylamine oxidation.

EXPERIMENTAL SECTION

Materials. Bovine liver mitochondrial MAO B was purified using the procedure outlined by Salach (1979) as further modified by Weyler and Salach (1981). Benzylamine, *N*-methylbenzylamine, DMABA, and *p*-(*N*,*N*-dimethylamino)benzaldehyde were purchased from Aldrich. α,α-Dideuterio-DMABA was synthesized by reduction of the corresponing nitrile (purchased from Aldrich) with $LiAlD_4$. NMDMABA was synthesized by borohydride reduction of the aldimine formed on the addition of methylamine to the *p*-(*N*,*N*-dimethylamino)benzaldehyde (Campbell et al., 1948). *N*,*N*-Dimethyl-*p*-(*N*,*N*-dimethylamino)benzylamine was synthesized by borohydride reduction of the aldimine formed on addition of dimethylamine to the aldehyde (Campbell et al., 1948). All synthetic amine analogues were recrystallized from ethanol as their hydrochloride salts. ^1H NMR spectral analysis of the synthetic amines in D_2O were obtained using a GE-QE300 instrument. The observed resonances for each compound were at the expected chemical shift values and exhibited integrated intensities consistent with their respective structures. All chemicals used were of reagent grade from commercial sources. Reduced Triton X-100 (Tiller et al., 1984) was purchased from Pearce Chemical Co.

Methods. Absorption spectral data were acquired using either a Cary 14R, a Hewlett-Packard diode array spectrometer, or a Milton-Roy diode array spectrometer. Anaerobic spectral measurements were performed under an Ar atmosphere in a custom quartz cuvette similar in design to that described by Williams et al. (1979). Stopped-flow absorbance kinetic measurements were performed using an apparatus purchased from Kinetic Instruments (Ann Arbor, MI). Time-dependent absorbance changes were collected using a Nicolet 4094 digital oscilloscope, and the kinetic data were transferred to an IBM XT computer via an IEEE488 interface for subsequent data analysis. Anaerobic stopped-flow measurements required deoxygenation of both the enzyme and substrate solutions by alternative pumping and flushing with purified Ar. The last traces of O_2 were removed by the addition (final concentrations) of glucose oxidase (22 nM), catalase (100 units/mL), and glucose (50 mM) to all solutions. Steady-state enzyme assays for benzaldehyde formation were performed spectrophotometrically (ϵ_M = 12 800 (Neumann et al., 1975)). Assays for the consumption of O_2 were performed polarographically using a Yellow Springs instrument equiped with a custom-made 2-mL thermostatable cell and a home-made voltage "backoff" device to allow full-scale chart recorder measurements of the initial 10% consumption of the O_2 concentration. All kinetic measurements were performed at 30 °C in 50 mM sodium phosphate and 0.5% (w/v) reduced Triton X-100 (pH 7.2).

Kinetic data analysis was performed using commercially available software. Single exponential decays were fit using MEDAS87 purchased from EMF Software (Knoxville, TN). Steady-state kinetic data were analyzed using Enzfiter (Elsevier), and nonlinear data were fit using MINSQ II (Micromath Scientific Software, Salt Lake City, UT).

RESULTS

Absorption Spectral Properties of Products Formed on MAO B-Catalyzed Oxidation. DMABA has an absorption maximum at 255 nm while *p*-(*N*,*N*-dimethylamino)benzaldehyde has a maximum at 352 nm. As shown in Figure 1, the addition of catalytic amounts of MAO B to DMABA results in the formation of an initial spectral species having

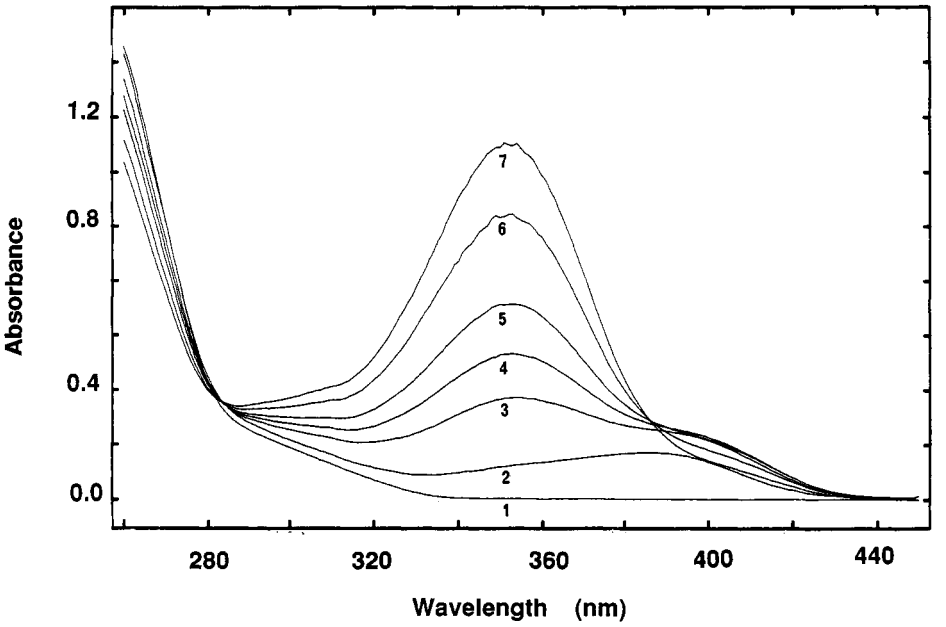


FIGURE 1: Spectral changes accompanying DMABA (149 μM) oxidation by MAO B in 50 mM sodium phosphate (pH 7.2) and 0.2% (w/v) reduced Triton X-100. Spectrum 1 is before enzyme addition. Spectra 2–7 were recorded at varying time intervals after the addition of 90 munits (measured as benzylamine oxidase activity at air saturation) of MAO B: 2, 5 min; 3, 16 min; 4, 24 min; 5, 35 min; 6, 59 min; and 7, 89 min.

an absorption maximum at 390 nm, which subsequently decays to *p*-(*N*,*N*-dimethylamino)benzaldehyde. A lag phase in the MAO-catalyzed formation of the aldehyde was observed previously by Deitrich and Erwin (1969) and is probably the result of formation of this spectral intermediate. Essentially identical spectral data are observed when MAO B is added to a solution of NMDMABA (i.e., an initial species formed with absorption at ~390 nm which subsequently decays to the aldehyde). When the spectral experiment (Figure 1) was performed at pH 9.1, no intermediate exhibiting an absorption at 390 nm was observed. As will be shown below, this effect of pH on the observability of this intermediate is due to the rapid hydrolysis of the imine product at pH 9.1 on its release from the enzyme. No formaldehyde was detected on analysis of the reaction products of NMDMABA oxidation using the Nash procedure (Werringloer, 1978), which shows oxidation to be exclusively at the benzyl carbon. This observation is in agreement with the finding of methylamine product formation upon the MAO-catalyzed oxidation of *N*-methylbenzylamine (Oi & Yasunobu, 1973). No spectral changes were observed on incubation of MAO B with *N*,*N*-dimethyl-*p*-(*N*,*N*-dimethylamino)benzylamine, which shows that this tertiary amine is not a substrate for MAO B although it does bind to the substrate site since it is found to be a weak competitive inhibitor of benzylamine oxidation (K_i = 2.25 mM).

These experiments show MAO B to oxidize either the benzylamine (primary amine) analogue or the *N*-methylbenzylamine(secondary amine) analogue but not the *N*,*N*-dimethylbenzylamine (tertiary amine) analogue. The formation of an initial spectral absorbing species with the two oxidizable substrate analogues, which subsequently decay to the same product (*p*-(*N*,*N*-dimethylamino)benzaldehyde), suggests that the identity of this intermediate is the DMABI which is initially released from the enzyme and subsequently hydrolyzes nonenzymatically to the aldehyde and to either NH_4^+ or $CH_3NH_3^+$ (depending on the substrate analogue used). To provide evidence for this hypothesis, we attempted to prepare DMABI by the procedure described by Ott (1981). Spectral analysis of solutions prepared from the crystalline product displayed one major absorption band at 350 nm. Rapid acidification of the neutral solution of DMABI (pH 7.5) to a final pH of 3.0 in stopped-flow pH-jump experiments resulted in the formation of a species with an absorption maximum at ~385 nm which subsequently decayed under the conditions of the experiments (data not shown). Similar experiments with *p*-(*N*,*N*-dimethylamino)benzaldehyde showed no detectable transient absorption at 385 nm. These data suggest that the 385-nm spectral species observed is due to the formation of the protonated form of the para-substituted benzenimine. Similar spectral data have been reported for the protonation of *p*-(*N*,*N*-dimethylamino)benzylhydrazone (Zverev et al., 1975).

Further support for these data is observed in static spectral experiments with NMDMABI. The addition of excess methylamine to an ethanolic solution of *p*-(*N*,*N*-dimethylamino)benzaldehyde results in spectral alterations consistent with imine formation (Figure 2a). Isosbestic points at 328 and 259 nm are observed, which document the presence of two species in solution. Note that the absorption maximum for the imine is at a *lower* wavelength than that of the aldehyde, which is a result of the "quinone" tautomeric structure which appears to be more favored for the aldehyde than for the imine. Protonation of the imine, however, results in a pronounced spectral shift to a maximum of ~390 nm (Figure 2B), which has the same absorption spectral properties as the spectral intermediate observed during the MAO B-catalyzed oxidation of either para-substituted benzylamine. Protonation of the imino nitrogen favors the quinone tautomeric structure to a degree higher than that observed for the aldehyde and accounts for the higher wavelength observed for the absorption maximum.

Incubation of NMDMABI in aqueous buffer results in slow hydrolysis to the aldehyde, which can readily be followed spectrophotometrically (Figure 3A). The rate of hydrolysis follows first-order kinetic behavior and is markedly increased at pH values above 6.5 (Figure 3B). The pH dependence of hydrolysis follows a single proton dependence (inset, Figure 3B), and a pK_a of 7.6 is estimated which corresponds to the pK_a of the imino nitrogen. Deprotonation of the imine

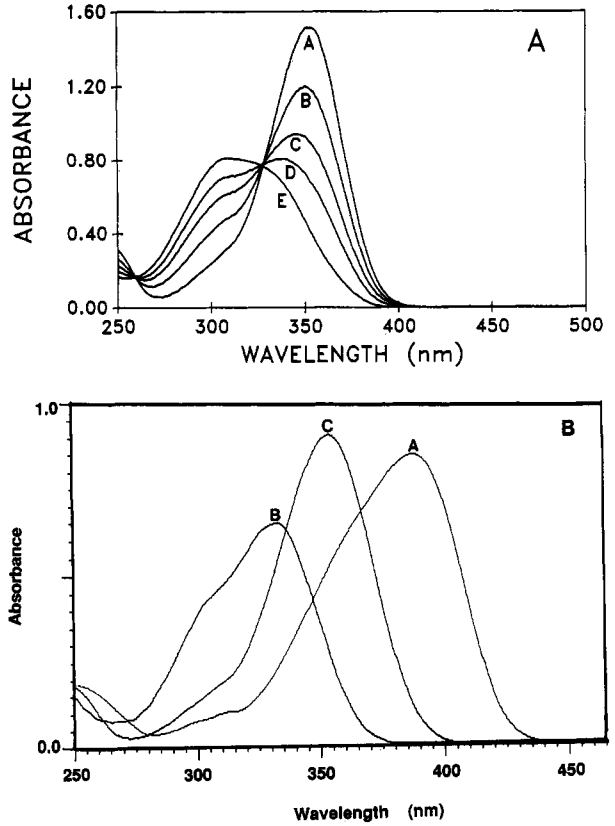
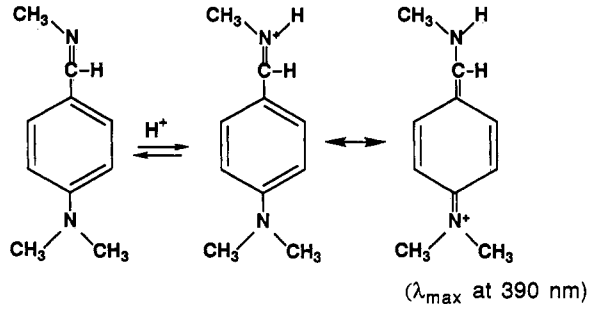


FIGURE 2: (A) Absorption spectral changes observed on the formation of NMDMABI after the addition of increasing concentrations of methylamine (free base) to 50 μM *p*-(*N*,*N*-dimethylamino)benzaldehyde in 100% ethanol: (A) before methylamine addition; (B–E) spectra after respective additions of methylamine (final concentrations) of 85, 169, 335, and 806 mM. (B) Absorption specta of protonated (spectrum A) and deprotonated (spectrum B) NMDMABI (24.3 μM) in 100% ethanol. Spectrum C is 30.6 μM *p*-(*N*,*N*-dimethylamino)benzaldehyde in 100% ethanol for comparison.

Scheme II



destabilizes the quinone tautomeric structure, and the resulting imine tautomeric form is more readily hydrolyzed.

Kinetic Properties with MAO B. To determine the catalytic constants of MAO B-catalyzed turnover of DMABA and NMDMABA, initial velocities were determined by monitoring O_2 consumption with an oxygen electrode. A turnover number of 1.4 s^{-1} and a K_m value of 1.2 mM was determined for NMDMABA at 0.9 mM O_2. By comparison, *N*-methylbenzylamine is oxidized by MAO B with a turnover number of 8 s^{-1} under the same conditions. Since the imine is predominant as the product for the *N*-methyl substrate at reaction times of less than 10 min, the rate of imine formation was determined spectrophotometrically at 390 nm as a measure of catalytic activity. At air saturation (~240 μM O_2), a turnover number of 1.5 s^{-1} and a K_m(amine) value of 0.8 mM were determined (data not shown). These results show good agreement between

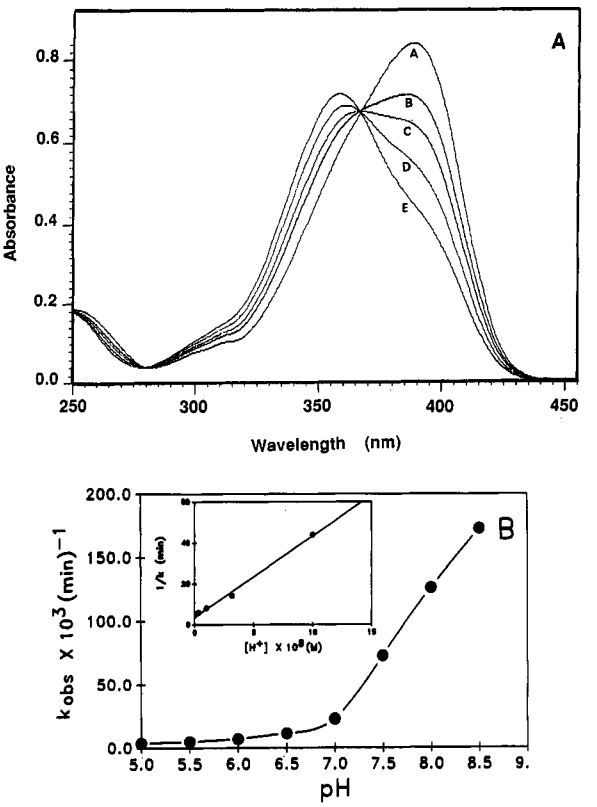


FIGURE 3: (A) Absorption spectral changes accompanying the hydrolysis of NMDMABI (24 μM) in 50 mM MES (pH 5.0) at various times after addition of imine to the buffer: (A) 0.5 min; (B) 30 min; (C) 50 min; (D) 80 min; and (E) 120 min. (B) Effect of pH on the rate of NMDMABI hydrolysis. In the pH range of 5.0–7.0, 50 mM MES buffer was used, and 50 mM EPPS buffer was used in the pH range of 7.5–9.0. Rates were determined spectrophotometrically from the initial rate of loss of absorption at 390 nm. The inset shows the dependence of the reciprocal observed rate on [H^+]. A pK_a of 7.6 is estimated from division of the slope value by the intercept value.

the two methods for assay and also demonstrate the K_m for O_2 to be lower than air saturation. Since the imine form of DMABA is less stable than the *N*-methyl analogue, rates of catalytic conversion by MAO B were determined at fixed substrate levels by the O_2 electrode assay. At 1 mM O_2 and 4 mM amine, a turnover number of 1.2 s^{-1} was determined, and it varied only slightly (1.0 s^{-1}) at 240 μM O_2 using 2 mM amine. Thus, the *p*-(*N*,*N*-dimethylamino)-substituted benzylamine is oxidized at ~10% the rate of benzylamine (Husain et al., 1982) at saturating substrate concentrations.

Stopped-Flow Kinetic Studies. The spectral properties of the imine products and the common aldehyde product from the MAO-catalyzed oxidation of these *p*-(*N*,*N*-dimethylamino)benzylamine analogues provide the possibility of monitoring the rates of imine formation as well as covalent flavin reduction by performing anaerobic half-reaction experiments. In accord with previous studies (Husain et al., 1982), the reaction is expected to follow this mechanism:

$$E\,FAD_{ox} + S \overset{K_s}{\rightleftharpoons} E\,FAD_{ox}{\cdot}S \xrightarrow{k_3} E\,FAD_{red}{\cdot}imine \longrightarrow E\,FAD_{red} + imine$$

The rate of conversion of E FAD_{ox} to E FAD_{red} can be followed spectrophotometrically at 450 nm, and the formation of imine from the amine can be followed at ~390 nm.

On mixing NMDMABA with MAO B under anaerobic conditions in the stopped-flow, the absorbance at 450 nm is

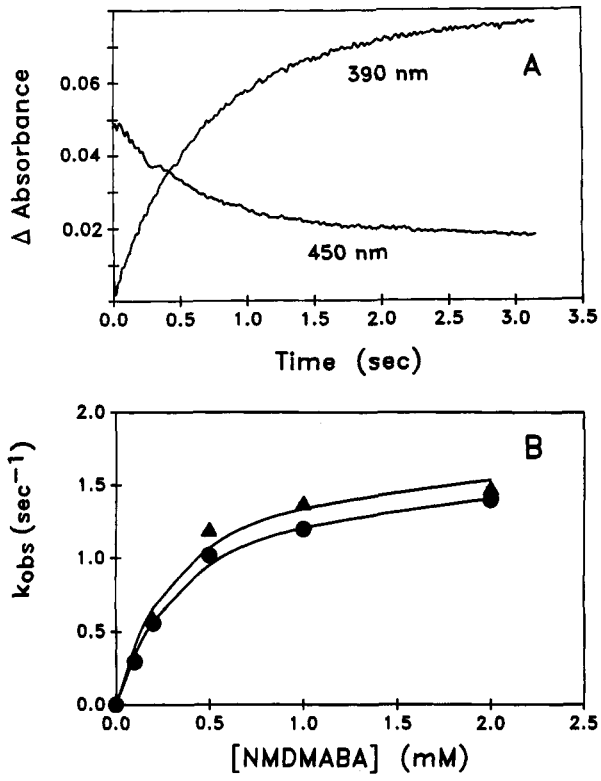


FIGURE 4: (A) Anaerobic reductive half-reaction observed by stopped-flow absorbance on mixing 4 μM MAO B with 2.0 mM NMDMABA (final concentrations). Enzyme-bound flavin reduction is measured at 450 nm, and protonated imine formation is monitored at 390 nm. (B) Effect of NMDMABA concentration on the observed rates of MAO B flavin reduction (▲) and protonated imine formation (●). The solid lines are from a nonlinear least-squares fit of the data point for the values of k_3 and K_s cited in the text.

bleached in a pseudo-first-order kinetic process corresponding to reduction of the covalent flavin to its hydroquinone form (Figure 4A). Concomitant with flavin reduction is an increase in absorption at 390 nm which we assign to formation of the protonated imine product. The rate of imine formation is identical with that of flavin reduction. As shown in Figure 4B, the limiting rate for flavin reduction (k_3 = 1.8 s^{-1}) is identical (within experimental error) with the value observed for imine formation (k_3 = 1.7 s^{-1}). Anaerobic reductive half-reaction experiments with MAO B and *N*-methylbenzylamine show a limiting rate of 17.3 s^{-1}, which is ~10-fold faster than the rate observed with NMDMABA. The K_s values determined for the concentration dependence of the two processes shown in Figure 4B are nearly identical (0.35 and 0.39 mM). The limiting rates for flavin reduction and imine formation are slightly faster than the rates of catalytic turnover determined spectrophotometrically at air saturation (1.5 s^{-1}) or polarographically at a high O_2 concentration (1.4 s^{-1}).

Under anaerobic conditions, the absorbance at 390 nm due to the protonated form of the imine product was found to decay very slowly. This behavior is demonstrated in the experiment shown in Figure 5. On addition of NMDMABA to MAO B, the difference absorption spectrum (obtained by subtraction of the spectrum of the oxidized enzyme before substrate addition) shows a negative absorption at 450 nm due to reduction of the flavin and an increased absorption at 385 nm due to the protonated imine product. (The difference absorption spectrum calculated from stopped-flow traces at different wavelengths is found to be identical with spectrum A in Figure 5). A slow, first-order loss of absorption at 390 nm is observed (k = 4.8 × 10^{-4} s^{-1}) (inset, Figure 5), which could be due to imine deprotonation and/or hydrolysis. The

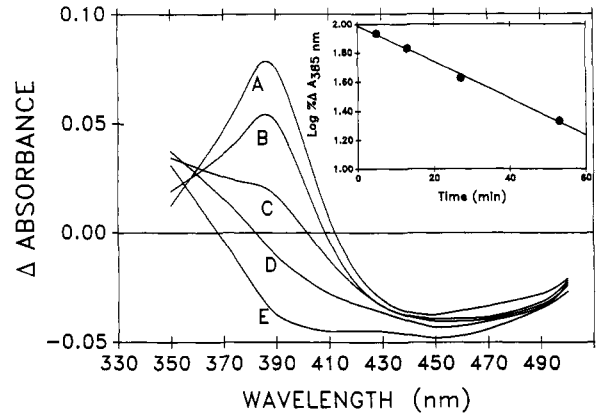


FIGURE 5: Difference absorption spectral changes occurring on the addition of 360 μM NMDMABA to 3.7 μM MAO B (final concentrations) under anaerobic conditions in 50 mM Na P_i and 0.5% (w/v) reduced Triton X-100 (pH 7.2). The difference spectra were generated by subtraction of the spectrum of the oxidized enzyme from those taken at various times after substrate addition: (A) 5 min; (B) 13 min; (C) 27 min; (D) 53 min; and (E) 113 min. The inset is a first-order plot of the change of absorbance at 385 nm with time. A first-order rate constant of 4.8 × 10^{-4} s^{-1} is calculated for this decay process.

rate observed is the same as that measured for the rate of imine hydrolysis (k = 3.8 × 10^{-4} s^{-1}) observed at the same pH in the absence of MAO B (Figure 3B). The rate of decay of the 390-nm absorption observed in Figure 5 (which occurs much slower than catalytic turnover) is interpreted to be due to imine product dissociation from the reduced enzyme and subsequent deprotonation and hydrolysis. From these data with NMDMABA, we conclude that O_2 reacts with the reduced-enzyme–imine product complex to form the oxidized-enzyme–imine product complex, which subsequently dissociates at a rate consistent with catalytic turnover as denoted in the bottom loop of the mechanism shown in Scheme I.

From the spectral data in Figure 2B, a $\Delta\epsilon_{352nm}$ of 4800 M^{-1} cm^{-1} is calculated for the extinction change for conversion of the protonated imine to the aldehyde. The absorption change observed at 352 nm on comparison of spectrum A with spectrum E corresponds to a stoichiometry of 0.96 mol of *p*-(*N*,*N*-dimethylamino)benzaldehyde/mol of MAO B flavin. A stoichiometry of close to 1 is expected since no turnover can occur in the absence of O_2.

It was of interest to determine whether the reaction of MAO B with DMABA also exhibits similar spectral and kinetic properties as observed with the *N*-methyl analogue. In anaerobic half-reduction stopped-flow experiments, the enzyme-bound flavin was reduced to its hydroquinone form, and the formation of protonated imine product was observed from an increase in absorption at 385 nm (data not shown). These kinetic transients followed pseudo-first-order kinetic behavior, and the observed rates were found to be dependent on the amine concentration. As shown in Figure 6, the limiting rate of flavin reduction (k_3 = 2.1 s^{-1}) is ~5-fold slower than the published rate of MAO B reduction by benzylamine (11.7 s^{-1}). Using α,α-dideuterio-DMABA, a k_H/k_D value of 10 is observed (Figure 6). In contrast with the results observed for NMDMABA (Figure 4), the limiting rate of protonated imine formation is found to be somewhat slower (k_3 = 1.3 s^{-1}) than the limiting rate of flavin reduction (Figure 6), but it exhibits an identical deuterium kinetic isotope effect (k_H/k_D = 9–10, data not shown). The K_s values for both kinetic processes are similar (0.5 mM for flavin reduction and 0.46 mM for protonated imine formation). At present, we have no definitive explanation for the differences in limiting rates

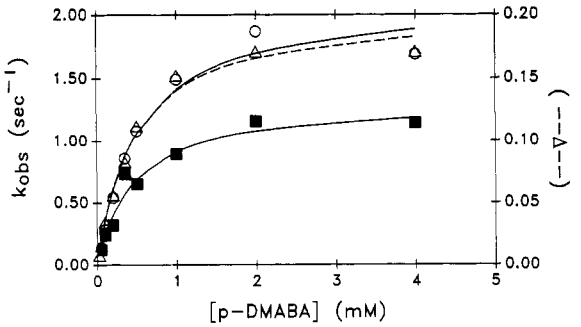


FIGURE 6: Effect of DMABA concentration on the observed rate of MAO B flavin reduction (○) and on the observed rate of protonated imine formation (■). The solid lines are for theoretical values of k_3 and K_s cited in the text. Effect of α,α-dideuterio-DMABA concentration on the observed rate (scale on right of the side of the figure) of MAO B flavin reduction (△). The dashed line is theoretical for a k_3 value of 0.2 s^{-1} and a K_s value of 0.46 mM.

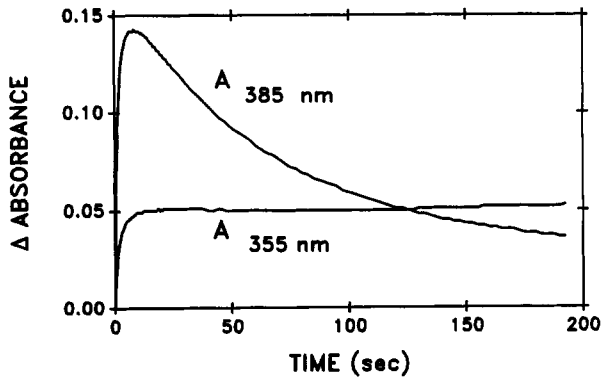


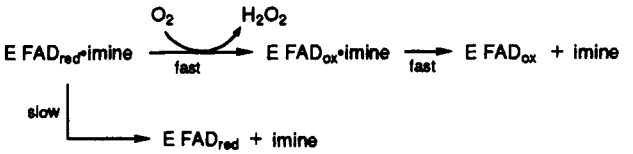
FIGURE 7: Absorbance changes monitored on mixing 2 mM DMABA and 4 μM MAO B under anaerobic conditions in the stopped-flow apparatus.

observed for the two kinetic processes. The slower rate of protonated imine formation might be an apparent result from prototropic equilibrium events occurring on imine formation, which would result in an apparent slower rate of protonated imine formation. Further work is required to clarify this aspect. Both rates, however, are comparable or slightly faster than turnover numbers determined at saturating substrate concentrations (see above).

In contrast to the slow decay of absorbance due to the protonated form of NMDMABA (Figure 5), the absorbance due to the imine product formed with DMABA decays more rapidly but at a rate slower than catalytic turnover (Figure 7). The rate of 385-nm absorbance decay is somewhat dependent on the amine concentration and shows a 7.6-fold increase in rate with an 80-fold increase in substrate concentration. This observed concentration dependence is probably due to the displacement of bound imine product from the reduced enzyme by excess substrate. In support of this suggestion, the k_H/k_D value for protonated imine decay is observed to be unity. Previous stopped-flow data on the interaction of MAO B with benzylamine suggest that the substrate can bind to the reduced form of the enzyme (Ramsey et al., 1987). The rate of decrease of the protonated amine absorbance *does not* correlate with the rate of aldehyde formation, which can be monitored at 355 nm. As shown in Figure 7, the absorbance at 355 nm remains constant during the time frame for loss of protonated imine absorbance. Thus, the decay of protonated imine is due to deprotonation rather than to hydrolysis. The faster rate of DMBI decay as compared with that of NMDMBI probably reflects the tighter binding of the latter product to the reduced enzyme.

DISCUSSION

The spectral and kinetic data presented in this article demonstrate the formation of a protonated imine as the initial product of the MAO-catalyzed oxidation of DMABA or NMDMABA. Imine formation and flavin reduction occur at rates consistent with catalytic turnover. The data presented for both benzylamine analogues suggest that amine oxidation to the imine results in a kinetically stable reduced-enzyme–imine complex which dissociates too slowly to be catalytically viable. During catalytic turnover, the reduced-enzyme–imine complex reacts with O_2 at a catalytically significant rate to form H_2O_2 and the oxidized-enzyme–imine complex.



The imine product must dissociate from the oxidized enzyme at a rate consistent with catalytic turnover and at a rate much faster than the rate of dissociation observed here from the reduced enzyme. The dissociated imine is then nonenzymatically hydrolyzed to the aldehyde and free amine or ammonium ion, as depicted in the bottom loop of the mechanism shown in Scheme I. Such a mechanistic scheme is necessary to account for the steady-state buildup of protonated imine shown in Figure 1. This mechanistic behavior has also been observed in the D-amino acid oxidase-catalyzed oxidation of D-α-aminobutyrate (Fitzpatrick & Massey, 1982). These results contrast with the suggestion by Tipton and co-workers (Houslay & Tipton, 1975; Tipton et al., 1987), who suggest that imine hydrolysis occurs on the enzyme and that release of NH_4^+ constitutes an irreversible step to account for the "ping-pong" kinetic behavior observed on steady-state analysis of MAO-catalyzed benzylamine oxidation. As pointed out by Palmer and Massey (1968) for the D-amino acid oxidase mechanism, parallel double-reciprocal plots are observed for the mechanism depicted in the bottom loop of Scheme I if k_3 is an irreversible step. This situation also appears to be the case with MAO B-catalyzed oxidation of benzylamines. It can also be concluded from these data that the use of DMABA as a substrate to monitor MAO B activity (Dietrich & Erwin, 1969; Tabakoff & Alivasatos, 1972) via aldehyde formation is complicated by the rate limitation of imine hydrolysis, the dependence of that rate on pH, and the nature of the buffer species used in the assay (Cordes & Jencks, 1963).

The processes for the transfer of reducing equivalents from the substrate to the flavin are either too rapid to be observed or any intermediates involved are at too low a concentration to be spectrally observable. Rapid freeze-quench ESR studies at times corresponding to maximal levels of imine formation using DMABA as substrate showed no detectable radicals at −150 °C, although the amount of MAO B used was high enough such that 10% formation of any radical would have been observed. The effect of *p*-(*N*,*N*-dimethylamino) substitution on the benzylamine substrate slows the rate of catalytic turnover and the rate of enzyme flavin reduction relative to the unsubstituted substrates by factors of 5–10 at saturating substrate concentrations. Similarly, the rate of enzyme reduction occurs at a rate similar to catalytic turnover, which suggests that enzyme reduction contributes to the rate limitation in catalytic turnover as observed previously for benzylamine (Husain et al., 1982) and is further supported

by the deuterium kinetic isotope effect observed in anaerobic reductive half-reaction experiments for the benzylamine substrate. The retardation of rate by the *p*-(*N*,*N*-dimethylamino) substituent could result from electronic effects (an electron-donating group), steric effects, and/or hydrophobic effects (Hartmann & Klinman, 1991). As will be documented in a separate publication (M. C. Walker, & D. E. Edmondson, manuscript in preparation), para substituent effects on the reduction of MAO B by benzylamine analogues are dominated by steric effects with little or no contribution from electronic or hydrophobic effects.

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