Mechanistic Studies on Dopamine β-Monooxygenase Catalysis: N-Dealkylation and Mechanism-Based Inhibition by Benzylic-Nitrogen-Containing Compounds. Evidence for a Single-Electron-Transfer Mechanism

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Abstract: Dopamine β-monooxygenase (DBM)[63] readily catalyzes oxidative N-dealkylation of *N*-phenylethylenediamine (PEDA) and *N*-methyl-*N*-phenylethylenediamine (*N*-MePEDA) with the reaction characteristics expected for a monooxygenase-catalyzed process. The products of this reaction have been quantitatively identified as aniline (or *N*-methylaniline for *N*-MePEDA) and 2-aminoacetaldehyde, the latter compound being successfully trapped by using $NaBH_4$ reduction followed by *N*-succinimidyl *p*-nitrophenylacetate (SNPA) derivatization, and identified by HPLC and mass spectroscopy. In contrast, either analogues of PEDA, i.e., phenyl 2-aminoethyl ether (PAEE) and its *p*-hydroxy derivative (*p*-OHPAEE), as well as 2-phenoxycyclopropylamine are not substrates but are competitive inhibitors. Furthermore, 2-methyl-2-anilino-1-aminoethane (β-MePEDA) did not exhibit measurable substrate activity with DBM, in contrast to the excellent substrate activity of the sulfur analogue of β-MePEDA, 2-methyl-2-(phenylthio)-1-aminoethane (β-MePAES). DBM is inactivated during the N-dealkylation reaction in a time- and concentration-dependent manner, a phenomenon that has not, to our knowledge, been observed for any other oxygenase-catalyzed N-dealkylation reaction. Both PEDA and *N*-MePEDA, as well as β-MePEDA, inactivate DBM under turnover conditions. The inactivation exhibited pseudo-first-order saturable kinetics and expected protection by the DBM substrate, tyramine. No reappearance of enzyme activity was observed after extensive dialysis. Radioactive labeling experiments with ring-tritiated PEDA showed incorporation of nondialyzable radioactivity into DBM in the expected amount, consistent with covalent attachment of a reactive species derived from PEDA to the DBM active site during enzyme inactivation. Although aniline, *N*-ethylaniline, *N*-(2-fluoroethyl)aniline, *m*- and *p*-anisidine, *p*-toluidine, and 5-hydroxyindole were found not to exhibit detectable DBM substrate activity, all of these inactivated the enzyme under turnover conditions. The isotope effect on partition ratio measured for dideuteriated PEDA was found to be a reflection of an isotope effect on V_{max} and not on k_{inact}. Our results provide a strong support for the conclusion that the initial nitrogen cation radical species is responsible for enzyme inactivation. Results with ring-deuteriated and ring-tritiated PEDA revealed that the amount of radioactivity incorporated into covalently inactivated DBM by ring-tritiated PEDA is in agreement with that expected for covalent attachment of the para carbon to the protein. An ^{18}O labeling study was carried out to test for oxygen rebound into the aminoacetaldehyde product, and the results demonstrated that the aldehyde oxygen of enzymatically produced 2-aminoacetaldehyde exchanges very rapidly with solvent water, in agreement with literature reports. On the basis of our results, a mechanism analogous to that currently favored for P-450-catalyzed N-dealkylations has been proposed herein for DBM-catalyzed N-dealkylation, a catalytic activity directly analogous to the peptidylglycine amidating monooxygenase reaction.

Dopamine β-monooxygenase [DBM; E.C. 1.14.17.1] a copper-containing monooxygenase present in mammalian tissues[1,2] catalyzes methylene hydroxylation at the benzylic position in a wide variety of substituted phenylalkylamines.[3] DBM has attracted increasing interest recently due to its key role in the biosynthesis of the adrenergic neurotransmitter, norepinephrine.[4,5] Accordingly, the mechanism of action of DBM must be critically considered in the design of novel neurotransmitter analogues and mechanism-based inhibitors of potential pharmacological interest. However, despite extensive investigations of DBM, fundamental questions regarding the mechanism of substrate and oxygen activation remain poorly understood.

Although DBM is traditionally viewed as a "specific hydroxylase", previous work in our laboratory has demonstrated several new kinetically facile monooxygenase activities for DBM: stereoselective sulfoxidation of phenylaminoalkyl sulfides,[6] oxygenative ketonization of benzylic S-alcohols,[7,8] selenoxidation of phenyl aminoethyl selenides,[9] and epoxidation of properly designed olefinic substrates.[10,11] In addition, DBM-catalyzed oxidative ketonization of halophenylethylamines has been reported by Klinman and co-workers,[12,13] and alkyne oxidation has been reported by us and by Villafranca and co-workers.[11,14] However, DBM is still considerably more selective than the relatively nonspecific cytochrome P-450 and microsomal flavin monooxygenases.

Cytochrome P-450, its model systems, and microsomal flavin-containing monooxygenases have long been known to catalyze N-, O-, and S-dealkylations.[15-17] In addition, an oxidative dealkylation activity for hemoglobin has been reported.[18] We have recently demonstrated that DBM readily catalyzes oxidative N-dealkylation of *N*-phenylethylenediamine (PEDA) and *N*-methyl-*N*-phenylethylenediamine (*N*-MePEDA), with the N-

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dealkylation activity exhibiting the stoichiometries of oxygen, electrons, and product diagnostic for a monooxygenase-catalyzed process.[11]

As stated in our initial report on DBM-catalyzed N-dealkylation[11] the rates of both oxygen consumption and product formation fall off rapidly with time, consistent with time-dependent inactivation of the enzyme. In the present paper we report on this process in detail and also on the related inhibition of DBM by aniline and its N- and ring-substituted derivatives. Our results conclusively establish that all these benzylic nitrogen containing compounds inhibit the enzyme in an apparent mechanism-based manner. All the data in hand strongly support a mechanism in which amine substrates undergo initial single-electron oxidation to generate a transient nitrogen cation radical intermediate, which partitions between enzyme inactivation and turnover. On the basis of our results and chemical and electrochemical precedence, we propose the nitrogen cation radical as the enzyme-inactivating species. In addition, a chemical mechanism that is analogous to P-450-catalyzed N-dealkylation is proposed herein for DBM-catalyzed N-dealkylation of PEDA-like compounds.

Experimental Section

Materials. Tyramine hydrochloride, ascorbic acid, and sodium fumarate were from Sigma. Phenylethylenediamine (PEDA), *N*-ethylaniline, aniline, and all substituted anilines were from Aldrich, and HCl salts were crystallized from EtOH/Et_2O. Tritiated water was from New England Nuclear. $H_2{}^{18}O$ was from MSD isotopes (97 atom % ^{18}O). Beef liver catalase (sp activity 65 000 units/mg of protein) was obtained from Boehringer Mannheim. Other chemicals and solvents were purchased from various sources and were of the highest purity available. HPLC analysis were performed on an LDC Constametric III system with an LDC Spectromonitor II Model 1204D variable wavelength detector. HPLC-MS analyses were kindly carried out by Dr. R. Thomas Solsten, Monsanto Company, Chesterfield, MO 63198, on a Finnigan 4500 Quadrapole Mass Spectrometer with a Vestac thermospray interface operating in the discharge ionization mode.

DBM Isolation and Assay. Dopamine β-monooxygenase was isolated and purified from bovine adrenals according to the procedure of Ljones, Skotland, and Flatmark,[19] with minor modifications, and exhibited a specific activity of 26–40 units/mg. (Unit is defined as 1 μmol/min of oxygen consumption with 10 mM tyramine as substrate in the standard oxygen monitor assay). The concentration of purified DBM tetramer [MW 290 000 (2)] was estimated spectrophotometrically by using E_{280} = 12.4. Kinetic constants (appk_{cat} and K_M) of substrates with DBM were determined by using the polarographic oxygen monitor assay and were calculated by computer fit of the data to the hyperbolic form of the Michaelis–Menten eq.[20] The standard DBM assay mixture, utilizing a YSI Model 53 polarographic oxygen monitor, contained 0.12 M sodium acetate buffer, pH 5.0 (unless otherwise stated), in the presence of 10 mM sodium fumarate, 0.10 mg/mL catalase, 0.5 μM $CuSO_4$, and 10 mM ascorbic acid, and 4–7 μg of DBM, at atmospheric oxygen saturation (unless otherwise stated) in a total volume of 2.5 mL. In our assay system, atmospheric oxygen saturation was found to be 250 μM.[21] Enzymatic reactions were initiated with substrate unless otherwise noted, and the initial rate was measured as the rate of oxygen consumption minus the small background ascorbic acid autooxidation rate.

Identification and Quantitation of Aromatic Products from the DBM/PEDA Reaction. The enzymatic reaction (150 μL) was quenched with 20 μL of concentrated HCl and filtered, and 15-μL samples were analyzed by HPLC. The HPLC analysis were carried out on a C-18 reverse phase column (10 cm, 5-Å pore, low load) with 65% 0.10 M NaOAc, pH 5.83, 5 mM sodium octylsulfonate (SOS), and 35% MeOH (v/v) as a mobile phase at a flow rate of 1.0 mL/min. The dealkylated aniline product was monitored by UV at 250 nm and was quantitated by using a standard curve based on the peak height. Identical procedures and conditions were used for the identification and quantitation of the N-dealkylated product of *N*-MePEDA, *N*-methylaniline.

Identification and Quantitation of 2-Aminoacetaldehyde from the DBM/PEDA Reaction. For each experiment the assay mixture contained 0.05 M NaOAc buffer at pH 5.6, 10 mM sodium fumarate, 10 mM ascorbate, 0.5 μM $CuSO_4$, 0.2 mg/mL of catalase, and 10 mM PEDA in a total volume of 1.0 mL. The enzymatic reactions were initiated by adding 50 μg of purified DBM and incubating at 37 °C for 6 min. After that period 200 mmol (100 μL of 2 M solution in 50 mM NaOH) of $NaBH_4$ was added, the reaction mixture was stirred for 1 h, pH was adjusted to 7.0, and 20 mg of SNPA in 1.0 mL of THF was added. After pH readjustment to 7.0, the reaction was allowed to stand for 45 min. Excess SNPA was destroyed with Na_2CO_3, and HPLC analysis was performed on a C-18 reverse phase column with 55% water and 45% methanol as mobile phase. The derivatized product of 2-aminoethanol was detected at 254 nm and quantitated by using a standard curve generated with authentic 2-aminoethanol. The control experiments (minus ascorbate and minus DBM) were treated exactly as described for the enzymatic reaction and analyzed by HPLC under identical conditions.

Time-Dependent Inactivation of DBM. Inactivation reactions, performed at 31 °C, contained 0.125 M sodium acetate buffer pH 5.0 (or 5.6), 9–10 mM sodium fumarate, 0.5 μM $CuSO_4$, 0.275 mg/mL of catalase, 10 mM ascorbic acid, and the indicated amount of DBM in a 0.40 mL total volume under atmospheric oxygen saturation conditions (unless otherwise stated). Inactivation was initiated by adding a required amount of inhibitor. The activity of DBM vs. time was assayed by the dilution of 40-μL aliquots of the inactivation mixture into the standard DBM assay mixture (2.5 mL of total volume). First-order inactivation constants were determined by linear regression analysis as the slopes of lines (k_{obsd}) resulting from plots of ln (% activity remaining) vs. time. Plots of $1/k_{obsd}$ vs. 1/[inhibitor] were linear and were fitted by linear regression analysis, with k_{inact} calculated as 1/(y-intercept) and K_I as −1/(x-intercept).

PEDA Radioactive Labeling of DBM. The inactivation solution contained 0.125 M NaOAc buffer at pH 5.6, 10 mM sodium fumarate, 0.5 μM $CuSO_4$, 44 mM ascorbate, 43 mM ^{3}H-PEDA (sp activity of 0.3 Ci/mol), 260 μg/mL of catalase, and 3.85 mg of purified DBM (sp activity 40) in a total volume of 2.70 mL. The enzymatic reaction mixture was stirred at room temperature for 1.5 h. (No enzyme activity was detectable in an identical parallel inactivation reaction after 30 min). The inactivation reaction mixture was loaded on to a Con-A Sepharose affinity column and was washed with 50 mM phosphate buffer (pH 6.5) containing 200 mM NaCl until no protein (A_{280}) or radioactivity was detected in the eluent. Separate control experiments revealed that catalase is not retained by Con-A Sepharose under these conditions. Inactivated DBM was eluted from Con-A Sepharose by stirring with 10 mL of 0.50 M methyl α-D-mannoside (in 50 mM phosphate buffer, pH 6.5, containing 200 mM NaCl), concentrated, and dialyzed in an ultrafiltration cell. The protein concentration (A_{280}, 60% recovery) and the radioactivity of the retentate were measured. A control experiment was carried out with no ascorbate under identical conditions.

^{18}O-Labeling Experiment. Incubation mixtures contained 0.10 M NaOAc at pH 5.6, 10 mM sodium fumarate, 0.5 μM $CuSO_4$, 100 μg/mL of catalase, 30 mM ascorbate, 20 mM PEDA, and 0.64 mg of purified DBM in a final volume of 1.00 mL of $H_2{}^{18}O$, 48.5% enriched in oxygen-18. The reaction was incubated for 7 min at room temperature, and 0.4-mL aliquots of incubate were added separately into two vials containing 0.4 mL of 97.0% ^{18}O enriched water and 0.4 mL of ^{16}O water. These two samples and the remaining portion of the incubate (0.2 mL) were stirred for 3 min at room temperature. $NaBH_4$ (200 μmol) was then added to each vial, and stirring was continued for an additional 30 min. The products from each reaction mixture were derivatized with SNPA and extracted with THF by saturating the reaction mixtures with NaCl. Extracts were dried in vacuo at room temperature, residues were taken up in methanol, and corresponding products were analyzed by HPLC-MS for ^{18}O content. Distribution of ^{18}O label in SNPA-derivatized 2-aminoethanol was calculated from relative intensities of (M^+ + H^+) peaks at 225 and 227 in positive ion current mass spectrum.

Synthesis. Synthesis of Radiolabeled PEDA. Radiolabeled PEDA was prepared by acid-catalyzed aromatic proton exchange, following the method of Muccino and Serico.[22a] Tritium oxide (1.0 mL, 25 mCi, 0.45 Ci/mol) was vacuum transferred into freshly distilled acetyl chloride (3.14 g, 40 mmol) cooled with dry ice–acetone. While still under vacuum, the mixture was transferred into another flask containing PEDA (400 mg, 3.0 mmol, free base). The reaction mixture was adjusted to atmospheric pressure (by adding argon), warmed up to 50 °C, and stirred for 24 h. Vacuum transfer removal of the [^{3}H]acetic acid and tritium chloride mixture gave a brown residue which was taken up in MeOH (3 × 4 mL, to remove labile tritium) and reconcentrated in vacuo. The crude product was dissolved in water, basified, and extracted with ether. The solvent was removed under reduced pressure. The HCl salt of the product was recrystallized several times with absolute EtOH and ether (300 mg, 1.7 mmol, 0.3 Ci/mol). HPLC analysis of this product gave a single peak with a retention time identical with that of cold PEDA and had 99% of the total radioactivity.

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N-Methyl-N-ethylenediamine (N-MePEDA). A solution of N-methyl-N-phenylethanolamine (7.0 g, 46 mmol) and tosyl chloride (10.0 g, 52.5 mmol) in Et_3N (50 mL) was stirred for 2 h at room temperature, refrigerated for 24 h, diluted with Et_2O, filtered, and evaporated to dryness. The crude tosylate derivative (12 g, 86%, 40 mmol) was dissolved in MeOH, and the resulting solution was saturated with $NH_3(g)$, stirred for 2 h at 0 °C followed by 2 h at room temperature. The reaction mixture was evaporated to dryness, dissolved in H_2O adjusted to pH 13, and extracted with Et_2O. The extracts were dried, filtered, and evaporated to yield N-MePEDA free amine (4.5 g, 75%). The hydrochloride was crystallized from EtOH/Et_2O to give a white solid, mp 199–200 °C (lit. 166 °C): ^1H NMR (D_2O) δ 7.63 (m, 5 H), 4.07 (m, 2 H), 3.37 (s, 3 H), 3.27 (m, 2 H). Anal. Calcd for $C_9H_{14}N_2$·HCl C, 57.90; H, 8.10; N, 15.01. Found: C, 57.85; H, 8.15; N, 15.05.

(±)-2-Methyl-2-anilino-1-aminoethane (β-MePEDA). A mixture of potassium phthalimide (18.5 g, 0.1 mol), chloroacetone (9.3 g, 0.11 mol) in 250 mL of DMF was stirred at room temperature for 1 h and then heated to reflux for 2 h under argon. The reaction mixture was cooled to room temperature, and the white solid was filtered and discarded. The filtrate was evaporated under reduced pressure to yield a yellow brown solid. This was crystallized from hot water to yield N-(2-oxopropane)-phthalimide (18.3 g, 90%) [^1H NMR ($CDCl_3$) δ 8.05–7.53 (m, 4 H), 4.53 (s, 2 H), 2.27 (s, 3 H)]. A mixture of aniline (3.72 g, 40 mM), N-(2-oxopropane)phthalimide (5.28 g, 26 mmol), $NaBH_3CN$ (1.15 g, 18.2 mmol), and methanolic HCl (2.7 mL of 5 N methanolic HCl, 13.5 mmol) in 350 mL of MeOH was stirred for 12 h at room temperature. Then the reaction mixture was concentrated under reduced pressure at room temperature and dissolved in water, and the pH was adjusted to 12–13 with KOH pellets and extracted with ether. The combined ether extracts were dried over anhydrous $MgSO_4$, and the HCl salt of 2-methyl-2-anilino-1-aminoethanephthalimide was precipitated by dropwise addition of methanolic HCl. This was crystallized from MeOH/Et_2O (4.6 g, 65%): ^1H NMR ($CDCl_3$, free base) δ 8.00–7.50 (m, 4 H), 7.40–6.43 (m, 5 H), 4.00 (m, 3 H), 1.43 (d, 3 H).

A solution of 2-methyl-2-anilino-1-aminoethanephthalimide (4.6 g, 17 mmol) and hydrazine hydrate (0.55 g, 17 mmol) in 100 mL of MeOH was stirred at 60 °C for 3 h. Then 10 mL of concentrated HCl was added and refluxed for 3 h. The reaction mixture was cooled to room temperature, filtered, and concentrated under reduced pressure to yield a white solid. The solid was dissolved in absolute ethanol, filtered, and evaporated to dryness. The resultant white solid was redissolved in water, adjusted to pH 5.0 with 6 N NaOH, and extracted with ether, and the ether layer was discarded. Then the aqueous layer was basified to pH 13 and extracted with ether, and the HCl salt of β-MePEDA was crystallized from EtOH/Et_2O (1.87 g, 59%): mp 199.5–200.5 °C; ^1H NMR (D_2O) δ 7.50 (s br, 5 H), 4.33–3.83 (m, 1 H), 4.3 (d, 2 H), 1.56 (d, 3 H); MS (EI), *m/e* 150 (M^+), 120 (100). Anal. Calcd for $C_9H_{14}N_2$·2 HCl: C, 48.44; H, 7.23; N, 12.55. Found: C, 48.45; H, 7.24; N, 12.51.

Phenyl 2-Aminoethyl Ether (PAEE). A mixture of phenol (14.0 g, 0.20 mol), KOH pellets (14.0 g, 0.25 mol), chloroacetonitrile (14 mL, 0.21 mol), and MeOH (200 mL) was stirred under N_2 for 24 h at room temperature. The reaction mixture was poured into ice-cold water and extracted with Et_2O, and the Et_2O extracts were washed with 0.1 N NaOH and brine, then dried, filtered, and evaporated to give phenoxyacetonitrile (15.2 g, 59%): ^1H NMR ($CDCl_3$) δ 7.50–6.80 (m, 5 H), 4.4 s, 2 H).

A mixture of phenoxyacetonitrile (13.3 g, 0.10 mol), $LiAlH_4/AlCl_3$ (0.10 mol each), and anhydrous Et_2O (300 mL) was refluxed for 3 h under nitrogen. The excess $LiAlH_4$ was destroyed by careful addition of H_2O, filtered, and dried over $MgSO_4$, and evaporated to give phenyl 2-aminoethyl ether (PAEE) free amine, which was crystallized as the HCl salt from EtOH/Et_2O: mp 221–222 °C (lit.[22b] 210 °C); ^1H NMR (D_2O) δ 7.00–7.65 (m, 5 H), 3.50 (t, 2 H), 4.40 (t, 2 H). Anal. Calcd for $C_8H_{11}NO$·HCl: C, 55.34; H, 6.97; N, 8.06. Found: C, 55.50; H, 7.01; N, 8.03.

p-Hydroxyphenyl 2-Aminoethyl Ether (p-OHPAEE). p-Hydroxyphenoxyacetic acid (Pfaltz and Bauer) was esterified with methanolic HCl in quantitative yield. The methyl ester was recrystallized from $CHCl_3$/hexane [^1H NMR (acetone-d_6) d, 6.80 (s, 4 H), 4.30 (s, 2 H), 3.70 (s, 3 H)]. The ester (3.0 g, 16.5 mmol) was dissolved in concentrated NH_4OH, stirred overnight at room temperature, and then evaporated under reduced pressure to give the amide derivative (2.3 g, 83%), which was used without further purification. The amide (2.3 g, 13.8 mmol) was suspended in dry THF (100 mL) and added to a suspension of $LiAlH_4$ (2.0 g, 52.6 mmol, in 50 mL of dry THF) dropwise, under N_2. The reaction mixture was refluxed for 12 h and cooled to room temperature, and water was cautiously added, followed by acidification with concentrated HCl and filtration. The aqueous layer was washed with Et_2O, concentrated, filtered and evaporated to dryness under reduced pressure. The resultant product, 4-OHPAEE HCl salt, was crystallized from EtOH/Et_2O, mp 172–174 °C dec (0.81 g, 31%): ^1H NMR (D_2O) δ 6.93 (s, 4 H), 4.25 (t, 2 H), 3.42 (t, 2 H).

2-Phenoxycyclopropane Carboxylic Acid. This compound was synthesized by the published procedure of Finkelstein et al.[23] A mixture of phenyl vinyl ether, (17.1 g, 0.14 mmol) 0.2 g of Cu powder, and 50 mL of dry xylene was stirred and heated to 120 °C. Then a solution of ethyl diazoacetate (Aldrich) (23.1 g, 0.20 mol) in 100 mL of dry xylene was added dropwise and at such a rate as to maintain the reaction temperature and to avoid a too vigorous reaction. When the nitrogen evolution ceased, the reaction mixture was refluxed for 1 h. The solvent was evaporated, and the red oil was distilled through a 30-cm Vigreux column and 112–120 °C (1 mm) fraction was collected: yield 12.3 g (42%). This was dissolved in 30 mL of ethanol containing 4 g of NaOH in 50 mL of water and refluxed for 12 h. The reaction mixture was cooled to room temperature, and most of the methanol was removed under reduced pressure. The residue was dissolved in water and acidified with dilute HCl to produce a crystalline product. This product was recrystallized from water to yield pure 2-phenoxycyclopropane carboxylic acid (a 70:30 mixture of trans:cis isomers,[23] 6.3 g, 22% overall): ^1H NMR acetone-d_6 δ 7.65–6.80 (m, 5 H), 4.20 (m, 1 H), 2.10 (m, 1 H), 1.42 (m, 2 H).

2-Phenoxycyclopropylamine. 2-Phenoxycyclopropylcarboxylic acid was converted to the corresponding amine by the method of Weinstock.[24] A mixture of 2-phenoxycyclopropylcarboxylic acid (3.0 g, 17 mmol), 5.0 mL of water, and 150 mL of acetone was cooled to 0 °C (ice–salt bath), and Et_3N (2.04 g, 20 mmol) in 38 mL of acetone was added. While maintaining the solution temperature at 0 °C, a solution of ethyl chloroformate (2.5 g, 26 mmol) in 9 mL of acetone was added slowly. The mixture was stirred for 30 min at 0 °C, and then a solution of NaN_3 (1.72 g, 26 mmol) in 6 mL of H_2O was added dropwise. The mixture was stirred at 0 °C for 1 h and poured into excess of ice water, and the red oil which separated was extracted into ether. The combined ether extracts were dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure at room temperature. The dark red oil (azide) was dissolved in 15 mL of anhydrous toluene and heated on a steam bath until no more N_2 was evolved. The dark red oil (isocyanate) was suspended in 25 mL of 20% HCl and refluxed overnight. Evaporation of the resulting solution gave a dark solid, which was crystallized from CH_3CN/Et_2O (1.2 g, 41%): mp 193 °C dec (lit.[23] cis isomer 189–191 °C, trans isomer 210–212 °C); ^1H NMR (D_2O) δ 7.52–6.85 (m, 5 H), 4.20 (m, 1 H), 3.00 (m, 1 H), 1.44 (m, 2 H); MS (EI), *m/e* 149, (M^+), 56 (100), (CI) *m/e* 150 ($M^+ + H^\bullet$).

(±)-2,5-Dimethyloxazoline. The procedure developed by Meyers et al.[25] was used with minor modifications. A mixture of ethyl acetamidate hydrochloride (20.3 g, 0.165 mol) and dry CH_2Cl_2 was cooled in an ice bath, and 1-amino-2-propanol (10.3 g, 0.137 mol) in 25 mL of dry CH_2Cl_2 was added dropwise. The reaction mixture was stirred for 6 h at 0 °C under nitrogen and was then poured into excess ice water. The organic layer was separated, and the aqueous layer was extracted twice with 50 mL of CH_2Cl_2. The combined extracts were dried over anhydrous $MgSO_4$, and solvent was removed under reduced pressure at room temperature to yield (12.0 g, 73.5%) a pale yellow liquid: ^1H NMR (neat) δ 4.70–2.85 (m, 3 H), 1.75 (d, 3 H), 1.10 (s, 3 H).

(±)-N-(2-Methyl-2-(phenylthio)-1-aminoethyl)acetamide. A mixture of (±)-2,5-dimethyloxazoline (8.0 g, 81 mmol) and thiophenol (8.9 g, 81 mmol) was heated at reflux for 48 h under nitrogen. Then the reaction mixture was cooled to room temperature and poured into ice-cooled water, and the oily product which separated was extracted with benzene. The combined benzene extracts were washed with 1 N NaOH and with brine and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure to yield a yellow oil (15.2 g, 90%): ^1H NMR ($CDCl_3$) δ 7.0–7.6 (m, 5 H), 2.9–3.6 (m, 3 H), 2.00 (s, 3 H), 1.25 (d, 3 H).

(±)-Methyl-2-(phenylthio)-1-aminoethane (β-MePAES). A mixture of N-(2-methyl-2-(phenylthio)-1-aminoethyl)acetamide (3.0 g, 14 mmol) and 50 mL of 6.5 N HCl was refluxed under nitrogen for 15 h. After extraction with ether, the aqueous phase was evaporated to yield (2.5 g, 94%) β-MePAES hydrochloride, which was crystallized from CH_3CN/Et_2O: hygroscopic, mp 98–99 °C; ^1H NMR (D_2O) δ 7.53 (m, 5 H), 3.8–3.27 (m, 1 H), 3.17 (d, 2 H), 1.40 (d, 3 H); MS (EI), *m/e* 167, (M^+), 138 (100).

2-Fluoroethyl p-Toluenesulfonate. This compound was synthesized by the published procedure of Howell et al.[26] A mixture of 2-fluoroethanol

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Table I. DBM Substrate Reaction Kinetic Parameters[a]

substrate	appk$_{cat}$, s^{-1}	K_m, mM	K_I, mM
PEDA	21	9.1	
PEDA[b]	44	14.0	
N-MePEDA	11	5.4	
β-MePEDA	*c*	*c*	
β-MePAES	33	9.3	
α-MePAES[d]	29	19.1	
PAES[d]	56	17.2	
PAEE	*c*		8.2
p-OHPAEE	*c*		60

[a] Kinetic values were obtained under standard assay conditions by using DBM of 32–21 units/mg specific activity at 0.25 mM O_2 concentration; DBM tetramer MW of 290 000 was used in appk$_{cat}$ calculations, and all values were normalized to 21 units/mg specific activity for comparison purposes. [b] NaOAC buffer, 0.125 M, pH 5.5. [c] Within the sensitivity limits of the oxygen monitor, no detectable or very weak O_2 consumption in standard assay. [d] Previously reported ref 58.

(25.0 g, 0.4 mol) and pyridine (100 mL) was stirred in a three-necked, round-bottomed flask immersed in a dry ice–acetone bath (maintained at −9 °C), and *p*-toluenesulfonyl chloride (73.3 g, 0.4 mol) was added to it slowly. Stirring was continued for 6 h, and the mixture was stored in the refrigerator overnight. Hydrochloric acid was added until the pyridine smell was imperceptible, and the resultant mixture was extracted with ether. The combined extracts were washed successively with water, aqueous Na_2CO_3, and finally again with water. After drying over anhydrous $CaCl_2$, removal of ether gave crude 2-fluoroethyl *p*-toluenesulfonate, which was distilled under reduced pressure to yield (45 g, 52%) a colorless liquid: bp 128–129 °C (0.5 mm) (lit. 138.5–140 °C) (1 mm); ^{1}H NMR ($CDCl_3$) δ 2.42 (s, 3 H), 4.10–4.32 (dt, J = 26, J = 2.5 Hz, 2 H), 4.10–5.02 (dt, J = 52, J = 2.5 Hz, 2 H), 7.30–7.78 (dd, J = 8 Hz, 4 H).

***N*-(2-Fluoroethyl)aniline Hydrochloride.** As described in the literature,[27] to a stirred solution of NaH [(3.20 g, 72 mmol) a 60% suspension in mineral oil (prewashed with hexane)], in 20 mL of freshly distilled DMF, was added *N*-benzyloxycarbonylaniline[28a] (12.0 g, 53 mol) in 100 mL of DMF under nitrogen. The reaction mixture was stirred at room temperature for 30 min. A solution of 2-fluoroethyl *p*-toluenesulfonate (13.1 g, 60 mmol) in 20 mL of DMF was added and stirred at room temperature for 4 h. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was partitioned between water and methylene chloride. The organic layer was separated, washed with water, dried, and evaporated under reduced pressure to leave an orange oil weighing 12.0 g (80%).

A cooled solution of 60 mL of 30% HBr in glacial acetic acid was added rapidly to *N*-(benzyloxycarbonyl)-*N*-(2-fluoroethyl)aniline (12.0 g, 44.4 mmol) with stirring and cooling (−5 °C). The solution was stirred for 20 min, the cooling bath was removed, and stirring was continued for 60 min. The reaction mixture was poured into a well-stirred mixture of 100 g of Na_2CO_3, 300 mL of saturated $NaHCO_3$, and 200 mL of ether. The ether layer was separated, washed with water, and dried over $MgSO_4$, and the ether was evaporated under reduced pressure. The HCl salt of *N*-(2-fluoroethyl)aniline was crystallized and recrystallized from EtOH/Et_2O to afford 7.0 g (92%): mp 168.5–169 °C (lit.[28b] 171 °C); ^{1}H NMR (D_2O) δ 3.60–4.40 (dt, J = 48, J = 2.5 Hz, 2 H), 4.03–5.14 (dt, J = 68, J = 2.5 Hz, 2 H), 7.55 (s, 5 H); MS (EI), m/e 139 (M^+), 106 (100).

Results

Incubation of the prototype diamine substrate, *N*-phenylethylenediamine (PEDA), or its N-methyl derivative *N*-phenyl-*N*-methylethylenediamine (*N*-MePEDA) with purified DBM in the presence of an electron donor, either ascorbate or ferrocyanide, results in an enzyme-dependent consumption of both electrons and oxygen in the stoichiometry diagnostic of monooxygenase-catalyzed oxygenations.[11] It is evident from the data in Table I that the ascorbate-dependent N-dealkylation and sulfoxidation activities of DBM exhibit comparable kinetic parameters and proceed with comparable facility at atmospheric oxygen concentration.

While PEDA and *N*-MePEDA exhibit comparable substrate activities, β-MePEDA did not exhibit any substrate activity with DBM under normal assay conditions at pH 5.0. With high concentrations of DBM (50 μg/2.5 mL) or at higher pH (5.5), this compound did exhibit very weak substrate activity, but accurate kinetic constants could not be obtained. These observations are in sharp contrast to the situation in the sulfur-containing series of DBM substrates. The sulfur analogue of β-MePEDA, β-MePAES, was found to be an excellent substrate for DBM with appk$_{cat}$ of 33 s^{-1} and a K_m of 9.3 mM. As shown in Table I, these kinetic parameters are comparable to those observed for other sulfur-containing DBM substrates.

In addition to these diamine compounds, a number of monoamine compounds were tested for DBM substrate activity. Among these were aniline, *N*-ethylaniline, *N*-(2-fluoroethyl)aniline, *p*-hydroxyaniline, *p*- and *m*-anisidine, *p*-toluidine, and 5-hydroxyindole. Within the sensitivity of the oxygen monitor assay, these compounds exhibited very weak or no substrate activity with DBM even in the presence of high concentrations of oxygen. In addition, we have previously shown that incubation of DBM with phenyl-2-aminoethyl ether (PAEE), the oxygen analogue of PEDA, under normal reaction conditions did not result in significant consumption of oxygen or electrons.[11] Similarly, no substrate activity is observed with 2-phenoxycyclopropylamine. Thus, DBM does not exhibit detectable oxidative O-dealkylation activity under the normal N-dealkylation reaction conditions. Kinetic studies established that both PAEE and its hydroxylated analogue, *p*-OHPAEE, are in fact potent competitive inhibitors of DBM (Table I) and are thus obviously capable of binding at the active site.

On the basis of TLC and quantitative HPLC analysis, we have previously demonstrated that the aromatic products formed from DBM-catalyzed oxygenation of PEDA and *N*-MePEDA are aniline and *N*-methylaniline, respectively, and that product formation is strictly dependent on the presence of DBM and a reducing agent.[11] In order to determine oxygen/product stoichiometry throughout the time course, parallel monitoring of oxygen consumption and quantitative HPLC determinations of aniline production were carried out. As is evident from Figure 1, the time courses of product formation and oxygen consumption are stoichiometrically consistent throughout, within the accuracy of the experimental procedure. In addition, N-dealkylation of *N*-MePEDA is regiospecific (see Discussion), and no lag period of product formation is observed with both PEDA (Table I) and *N*-MePEDA, indicating that N-dealkylation is a direct result of substrate turnover. Furthermore, it is also clear from the data presented in Figure 1 that the rate of aniline formation is retarded in the presence of tyramine, as would be expected for reactions occurring at the same active site.

It was anticipated that the second product formed from dealkylation of either PEDA or *N*-MePEDA would be 2-aminoacetaldehyde. In agreement with literature reports,[29] we found this aldehyde to be very unstable under conditions suitable for trapping; experiments with authentic 2-aminoethanol indicated that *N*-succinimidyl-*p*-nitrophenyl acetate (SNPA) could be used to derivatize 2-aminoethanol in aqueous medium, and the derivative could be quantitatively detected by reverse phase HPLC. Accordingly, enzymatic reaction mixtures were subjected to $NaBH_4$ reduction followed by SNPA derivatization, and analysis by reverse phase HPLC showed the presence of a peak with the retention time identical with that of derivatized authentic 2-aminoethanol (Figure 2A). This identification was further confirmed by HPLC-MS analysis. Control experiments indicated that omission of DBM, ascorbate (Figure 2B), or PEDA abolishes the formation of 2-aminoacetaldehyde. Quantitation using an HPLC standard curve based on peak height yielded an aldehyde/aniline stoichiometry for the DBM/PEDA reaction of 0.7:1.0.

Despite our recognition that aldehydes readily exchange in aqueous media,[30] a series of experiments were carried out in the

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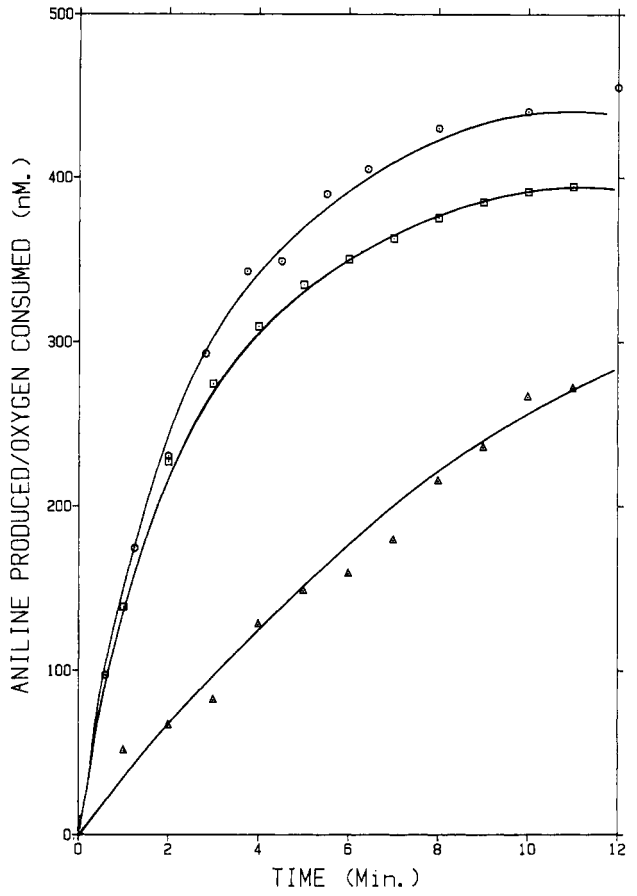

Figure 1. Time course of aniline formation and oxygen consumption in the presence and absence of tyramine during the N-dealkylation of PEDA by DBM. The reaction mixture contained 10 mM sodium fumarate, 0.5 μM $CuSO_4$, 0.10 mg/mL of catalase, 12 mM PEDA, 60 μg of purified DBM in 2.5 mL of 0.125 M NaOAC, pH 5.5. During each time interval a 150-μL sample was withdrawn and added into 20 μL of concentrated HCl and filtered, and 15-μL samples were analyzed by HPLC. The amount of aniline was determined as described in Materials and Methods: (⊡) nmol of oxygen consumption with no tyramine present; (⊙) nmol of aniline production with no tyramine present. (△) nmol of aniline production in the presence of 1.0 mM tyramine.

presence of $H_2{}^{18}O$ in an attempt to trace the source of oxygen in the aldehyde dealkylation product. The experiments were designed so that they would simultaneously provide information regarding the extent of exchange during enzymatic turnover and the manipulations necessary for product trapping. The experimental protocol involved two stages: turnover in the presence of $H_2{}^{18}O$, 48.5 enriched in oxygen-18, followed by a dilution and further incubation to allow exchange to proceed under defined solvent conditions. Following this, the aldehyde was reduced with $NaBH_4$ and derivatized with SNPA. After salting into THF, the solvent was evaporated, and the residue was dissolved in methanol and subjected to both HPLC and HPLC-MS analysis. The experimental design and expected results are illustrated in Table III, and Figure 2C illustrates the mass spectral data obtained from analysis of each reaction mixture.

The experimental results clearly show that exchange is rapidly occurring, since in every case enrichment deviates from the expectation value in the direction expected for exchange. Furthermore, the extent of exchange is almost total under the experimental conditions. Therefore, it is obvious that ^{18}O-labeling experiments could not be used to trace the source of aldehyde oxygen in DBM-catalyzed N-dealkylation. It is evident from Figure 2C that the mass spectral data provide unequivocal identification of 2-aminoacetaldehyde as a product of enzymatic processing of PEDA.

Table II. DBM Inactivation Kinetic Parameters

inhibitor	$appk_{inact}$,[a] min^{-1}	K_I, mM	$appk_{cab}$[b]/ $appk_{inact}$	$appk_{inact}/K_I$
PEDA	0.18	1.1	1750	0.16
PEDA[c]	0.41	2.0	1600	0.21
N-MePEDA	0.10	4.5	1650	0.02
β-MePEDA	0.10	5.3	60[d]	0.02
N-ethylaniline	0.46	6.8	*e*	0.07
N-(2-fluoroethyl)aniline	0.52	2.0	*e*	0.26
aniline	0.41	1.8	*e*	0.23
m-anisidine	0.22	1.2	*e*	0.18
p-anisidine	0.83	38.4	*e*	0.02
p-toluidine	0.81	2.1	*e*	0.35
5-hydroxyindole	0.16	1.5	*e*	0.11

[a] Determined as described in Materials and Methods, at four or more inhibitor concentrations. All k_{inact} and K_I values are apparent values obtained at a single oxygen concentration (0.25 mM). [b] For partition ratio calculations, the $appk_{cat}$ constants were calculated based on a DBM active site MW of 73 000. [c] NaOAC buffer, 0.125 M, pH 5.5. [d] Weak ascorbate-dependent DBM substrate activity was observed, calculated as total oxygen consumed at 100% inactivation/concentration of DBM. [e] Very weak substrate activity was observed, but the low activity made $appk_{cat}$ determination nonreproducible.

Examination of the time course of the oxygenation of PEDA or *N*-MePEDA reveals a rapid fall off in turnover rate with time (Figure 1). As shown in Figure 3A, the loss of enzyme activity under DBM/PEDA turnover conditions exhibits saturable, pseudo-first-order kinetics. In addition, it was found that inactivation is concentration dependent and also depends strictly on the presence of a reducing agent. By using the dilution assay method described in the Materials and Methods Section, first-order kinetic parameters were obtained, and the double reciprocal plot (Figure 3B) yielded the values of $appk_{inact}$ = 0.18 min^{-1} and K_I = 1.1 mM at 0.25 mM O_2 and pH 5.0 (Table II), assuming the simplest kinetic scheme for suicide inactivation.[31] Thus, the kinetic partition ratio for PEDA, $appk_{cat}/appk_{inact}$, is 1750 turnovers/inactivation. For this calculation, and for all partition ratios calculated herein, $appk_{cat}$ is based on a monomer MW of 73 000. The $appk_{inact}$ of PEDA is highly dependent on pH; thus, at pH 5.5 $appk_{inact}$ was found to be 0.41 min^{-1} and K_I = 2.0 mM. However, it is clear from the data presented in Table II that the kinetic partition ratios for PEDA are independent of pH. This result is consistent with a mechanism whereby both N-dealkylation activity and time-dependent inactivation proceed through at least one common acid/base-sensitive step.

Inactivation of DBM by PEDA goes completely to 100% inactivation (with 5.0 mM inhibitor concentration after 20 min), and no reappearance of enzyme activity was observed even after prolonged incubation with tyramine. Similarly, no reversal of inactivation was observed even after 24 h of dialysis against 0.1 M MES, pH 6.0, conditions under which the control (minus ascorbate) exhibited at least 40% of its original activity. Inclusion of the substrate tyramine at 20 mM concentration in the inactivation mixture protected against inactivation (Figure 3A), and exclusion of ascorbate completely abolishes the inactivation (data not shown), as expected.

To test whether the dealkylated products of the DBM/PEDA reaction can return to the active site and inactivate the enzyme independent of turnover, the experiment shown in Figure 4 was performed. As seen in Figure 4, the time courses of O_2 uptake and concomitant enzyme inactivations were identical between a standard reaction mixture and the same reaction mixture when fresh aliquots of DBM were added after 100% enzyme inactivation and reequilibration with air. If any moderately stable reactive species were released upon PEDA turnover, one would expect to see more rapid inactivation rates for the subsequent DBM aliquots. Examination of the time courses in Figure 4 shows that decay of O_2 consumption is identical with and without preformed reaction products present. However, as is noted below, one of the products of the PEDA N-dealkylation reaction, aniline, is also a time- and

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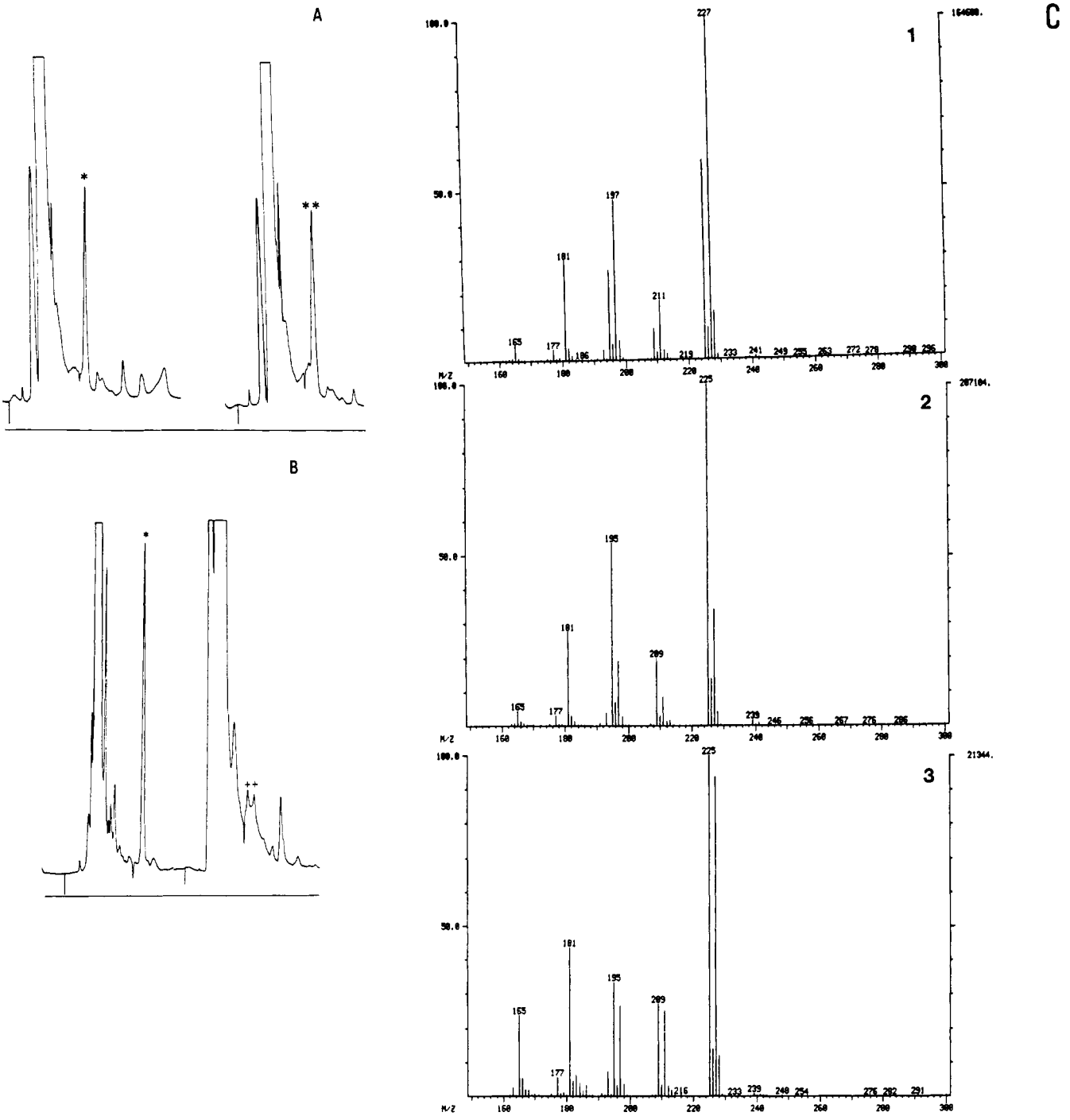

Figure 2. Identification of 2-aminoacetaldehyde as the second product from the DBM/PEDA reaction. Panel 2A: The assay mixture contained 0.05 M NaOAc, pH 5.6 buffer, 10 mM sodium fumarate, 10 mM ascorbate, 0.5 μM $CuSO_4$, 0.2 mg/mL of catalase, and 10 mM PEDA in a total volume of 1.0 mL. The enzyme reaction was initiated by adding 50 μg of purified DBM and incubated at 37 °C for 6 min. Then 10 mg of $NaBH_4$ was added, and the reaction mixture was stirred for 1 h. The pH of the reaction mixture was adjusted to 7.0, derivatized with SNPA, and analyzed by HPLC as described in the Experimental Section: (*) derivatized 2-aminoethanol from DBM/PEDA reaction; (**) enzymatic product spiked with derivatized authentic 2-aminoethanol. Panel 2B: (*) Same as above; (++) same as above except no ascorbate was present in the incubation mixture. Panel 2C: HPLC-MS analysis of $NaBH_4$-reduced, SNPA-derivatized 2-aminoacetaldehyde from DBM/PEDA reaction mixtures. Enzymatic incubations were carried out in $H_2{}^{18}O$, 48.5% enriched in oxygen-18. Enzymatic reaction mixtures were diluted and incubated with equal volumes of either (1), 97.0% ^{18}O enriched water; (2), ^{16}O water; or (3), undiluted. The product from each incubation mixture was derivatized and analyzed by HPLC-MS as described in Table III.

concentration-dependent inhibitor for DBM at much higher concentration.

In order to confirm that inactivation by PEDA involves covalent attachment of a substrate-derived species to the DBM active site, ring-tritiated PEDA was prepared and incubated with DBM under the conditions described in the Materials and Methods section. After total inactivation of the enzyme, the reaction mixture was loaded onto a Con-A Sepharose column. The column was washed until no more radioactivity or protein (catalase) was detected in the eluate, and inactivated DBM was eluted with methyl α-D-mannoside. Concentrated, inactivated enzyme was dialyzed in an ultrafiltration cell against 10 volumes of 10 mM BES, pH 7.0, after which no radioactivity was detected in the filtrate. The UV spectrum of the resulting material was identical with the native DBM, and no absorption peak at 400 nm was observed, indicating the absence of catalase contamination. Scintillation counting of the inactivated enzyme showed definite nondialyzable-radiolabel incorporation into the inactivated enzyme. Quantitation indicated

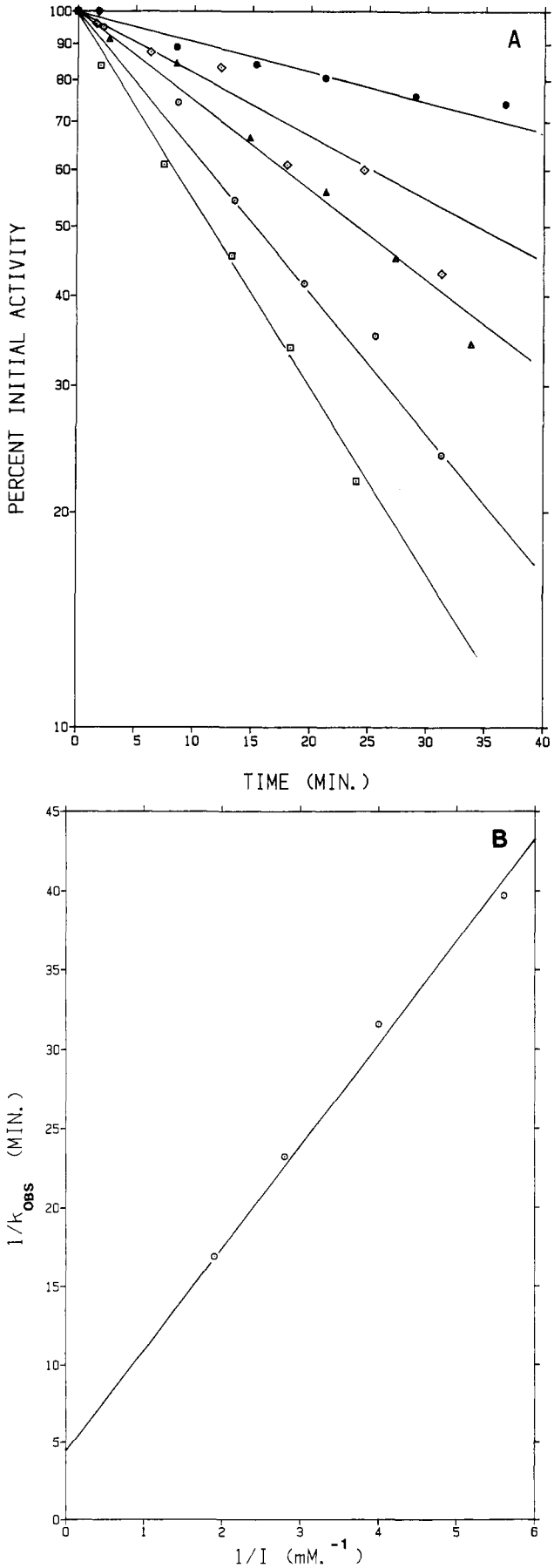

Figure 3. Kinetics of time dependent inactivation of DBM with PEDA. Panel 3A: (⊡) 0.537 mM; (⊙) 0.357 mM; (△) 0.250 mM; (◇) 0.179 mM; (●) 0.537 mM PEDA + 20 mM tyramine (conditions as described in the Materials and Methods Section). Panel 3B: double reciprocal-plot of data from Figure 3A.

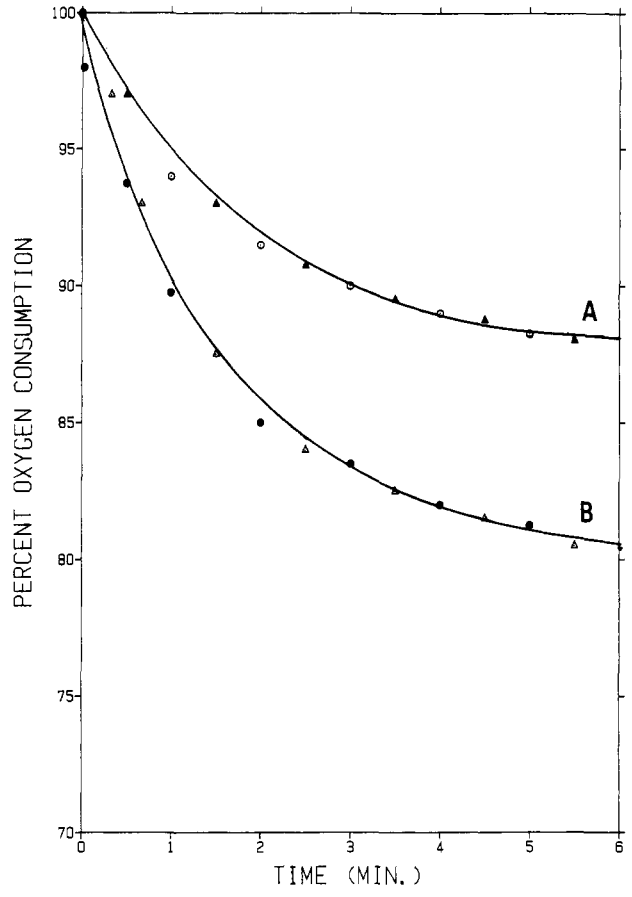

Figure 4. Time course of oxygen uptake and inactivation rate in the absence and presence of DBM/PEDA preformed products. The reaction mixture contained 10 mM fumarate, 0.25 mg/mL of catalase, 0.5 μM $CuSO_4$, 20 mM ascorbate, and 20 mM PEDA in 0.125 M NaOAc, pH 5.0 (2.575 mL is the total volume). The reaction, run at 37 °C, was initiated by adding 4.4 μg of DBM and was monitored by the standard dissolved oxygen electrode assay (curve A, ⊙). After total inactivation of the enzyme, the system was resaturated with air, an identical aliquot of DBM was added, and the reaction monitored (curve A, ▲). After total inactivation a third aliquot of DBM containing twice as much enzyme (8.8 μg) was added, and reaction was monitored (curve B, △). After total inactivation of the enzyme, the system was resaturated with air, and another identical aliquot (8.8 μg) of DBM was added (curve B, ●).

that the amount of radioactivity bound to the enzyme was 0.74 molecules of PEDA/enzyme subunit of M_r 73 000. Thus, the results conclusively established the covalent attachment of a reactive species derived from PEDA to the DBM active site during enzyme inactivation. In a separate control experiment, DBM was incubated with the same amount of tritiated PEDA with no ascorbate present, and less than 0.05 molecules of labeled PEDA were found to have been incorporated into the enzyme.

Inactivation of DBM with *N*-MePEDA is also an ascorbate-dependent, first-order process protected against by tyramine, which is slower than that observed for PEDA (Table II). The kinetic partition ratio for *N*-MePEDA is 1650, a value almost identical with that for PEDA. While β-MePEDA does not exhibit measurable substrate activity under normal assay conditions, it too is a time-, concentration-, and ascorbate-dependent DBM inhibitor exhibiting normal first-order kinetics and tyramine protection (Table II). Due to the extremely weak substrate activity of this compound, it was not possible to obtain a meaningful value for the kinetic partition ratio. However, an estimation based on total oxygen consumption before inactivation of a given amount of DBM yielded a value of 60 turnovers/inactivation, indicating that this compound is a much more efficient inhibitor than either PEDA or *N*-MePEDA.

As stated above, *N*-alkylanilines do not exhibit detectable substrate activity with DBM. In contrast, incubation of *N*-ethylaniline with DBM resulted in rapid loss of enzyme activity,

Table III. ^{18}O-Labeling Experiment

incubtn mixture[b] (final ratio of ^{18}O:^{16}O in mixture)	distribution of ^{18}O:^{16}O in product aldehyde[a]			
	expected			
	aldehyde O from atm O and no exchange	aldehyde O from H_2O and no exchange	in either case if exchange is 100%	exptl
1. 0.4 mL enzymtc[c] reactn mixture plus 0.4 mL ^{18}O H_2O (72.8:27.2)	3.0:97.0	48.5:51.5	72.8:27.2	63.1:36.9
2. 0.4 mL enzymtc reactn mixture plus 0.4 mL ^{16}O H_2O (27.2:72.8)	3.0:97.0	48.5:51.5	27.2:72.8	25.5:74.5
3. 0.2 mL enzymtc reactn mixture (48.5:51.5)	3.0:97.0	48.5:51.5	48.5:51.5	48.4:51.6

[a] The $NaBH_4$-reduced enzymatic product, 2-aminoethanol, from each experiment was derivatized with SNPA, salted out into THF, and dried under vacuo. The residues were taken up in methanol and analyzed by HPLC-MS as described in the Experimental Section. The ^{18}O:^{16}O ratio is based on the relative intensities of ($M^+ + H^+$) peaks at *m/e* 227 and 225. [b] The enzymatic reaction mixtures, diluted with equal volumes of either (1) 97.0% ^{18}O enriched water, (2) ^{16}O water, or (3) undiluted were incubated at room temperature for 3 min and reduced with $NaBH_4$ as indicated in the Experimental Section. [c] The enzymatic reaction mixture contained 0.10 M NaOAc, pH 5.5, 10 mM sodium fumarate, 0.5 μM $CuSO_4$, 100 μg/mL catalase, 30 mM ascorbate, 20 mM PEDA, and 0.64 mg of purified DBM in a final volume of 1.00 mL of $H_2{}^{18}O$ 48.5% enriched in oxygen-18. The reaction was incubated for 7 min at room temperature and treated as in footnote a.

which exhibited time-, concentration-, and ascorbate-dependent first-order saturable kinetics (Table II) and tyramine protection. In order to examine the effect of increased acidity of the C_α hydrogen on inactivation potency, *N*-(2-fluoroethyl)aniline was synthesized and examined as a DBM inhibitor (fluoro-substitution is not possible for PEDA itself). *N*-(2-fluoroethyl)aniline was found to be a potent inhibitor, exhibiting strictly ascorbate-dependent, first-order inactivation kinetics similar to that obtained for *N*-ethylaniline (Table II).

5-Hydroxyindole, where no abstractable hydrogen is available, was examined as a potential inhibitor for DBM. The dilution inactivation assay revealed that this compound is also a time-, concentration-, and ascorbate-dependent inhibitor for DBM, indicating that the presence of abstractable C_α hydrogen is not required for the enzyme inactivation. In view of these findings we wished to examine aniline itself as an inhibitor for DBM to determine whether the N-alkyl group plays an important role in the enzyme inactivation by benzylic-nitrogen-containing inhibitors at all. Aniline was also found to be a potent inhibitor for DBM which exhibited oxygen- and ascorbate-dependent first-order kinetics (Table II), tyramine protection, and nonreversal of enzyme inactivation after dialysis. In addition, *p*-toluidine and *p*- and *m*-anisidine were also found to be time-, concentration-, and ascorbate-dependent inhibitors for DBM (Table II). In contrast, *p*-hydroxyaniline was found to be an ascorbate-independent potent inhibitor for DBM and followed pseudo-first-order kinetics. Although no detailed studies were carried out on this phenomenon, this appears to be a complex process.

PEDA, dideuteriated at the β carbon, was synthesized and characterized, and the isotope effect on the partition ratio was measured and found to be 2.8 ± 0.1. Furthermore, as will be discussed elsewhere (unpublished results), the isotope effect on V_{max} is virtually the same as that measured for the partition ratio. These results are consistent with the view that while the complete N-dealkylation process obviously involves C–H bond cleavage at the adjacent carbon, the species responsible for inactivation is likely to be the cation radical generated by single-electron oxidation of the benzylic nitrogen prior to C–H bond cleavage. Such a cation radical could inactivate DBM via covalent attachment through its aromatic ring. Indeed, the stoichiometry of DBM/ring-tritiated PEDA shows that the amount of radioactivity incorporated into DBM by ring-tritiated PEDA is in agreement with what would be expected for covalent attachment via the para carbon, as is evident from the following results. Deuterium exchange into PEDA was carried out under the same conditions used for tritiation, and ring-deuteriated PEDA was isolated and subjected to mass spectral analysis. The mass spectrum showed that 20% of the deuterium label was in the para position, 72% in the ortho, and 8% in the meta position. Thus, covalent attachment via the para carbon of the aromatic ring should lead to loss of about 20% of the tritium label, as observed. Examination of the specific activity of the aniline produced during enzymatic turnover of tritiated PEDA indicated retention of 95% of the initial radioactivity, thus confirming that turnover alone does not lead to any appreciable loss of label from PEDA.

As stated above, the ether analogues of PEDA—PAEE and *p*-OHPAEE—are not substrates for DBM. Furthermore, neither of these two ethers nor 2-phenoxycyclopropylamine exhibits appreciable inactivation activities even at high oxygen concentrations. These results confirm the conclusion that DBM is catalytically inactive toward the ethers under the normal assay conditions.

Discussion

Monooxygenase-catalyzed N-dealkylation reactions are well-documented for heme- and flavin-containing proteins.[15-18] In contrast, non-heme metallo monooxygenase-catalyzed N-dealkylation reactions were unknown prior to our initial report on the substrate activity of PEDA.[11] It is striking that DBM-catalyzed N-dealkylation of benzylic-nitrogen-containing compounds proceeds with concomitant inactivation of the enzyme, a phenomenon which has not been reported for any other oxygenase.

Results reported herein clearly establish the identities of, and quantitate, both the aniline and the aldehyde products formed from DBM processing of the prototypical substrates, PEDA and *N*-MePEDA. Furthermore, the stoichiometries of oxygen, electrons, and products are fully consistent with a monooxygenase-catalyzed reductive oxygenative pathway. It is also important to note that N-dealkylation of *N*-MePEDA proceeds regiospecifically, producing only *N*-methylaniline and 2-aminoacetaldehyde, despite the possibility of N-demethylation to generate PEDA and formaldehyde as the reaction products. This fact reflects the specificity of the enzyme. It also eliminates the possibility that N-dealkylation is a result of a solution decomposition of a putative enzymatically generated *N*-oxide, since such a solution process would also generate PEDA and formaldehyde.[32]

Removal of the terminal amino group from PEDA to give *N*-ethylaniline dramatically decreases the substrate activity; the extremely weak substrate activity of *N*-ethylaniline made it impossible to obtain precise kinetic parameters. This is in accord with our observations on DBM-catalyzed hydroxylations, sulfoxidations, and olefin oxidations; in all cases the presence of a terminal amino group facilitates catalytic turnover. Similarly, no detectable substrate activity was observed with aniline, *N*-ethylaniline, *N*-(2-fluoroethyl)aniline, *p*-hydroxyaniline, *p*-toluidine, *p*- and *m*-anisidines, and 5-hydroxyindole.

In contrast to the competence of DBM to catalyze N-dealkylation, O-dealkylation was not observed even at high oxygen concentrations, despite the fact that the ethers examined are good competitive inhibitors and obviously do bind to the enzyme. The mechanistic significance of these findings becomes clear when considered in light of the current view of the mechanism of P-450-catalyzed reactions. While sulfoxidation and N-dealkylation activities of P-450 are generally viewed as proceeding through the intermediacy of a species with cationic and/or radical character, generated by initial abstraction of a single electron from

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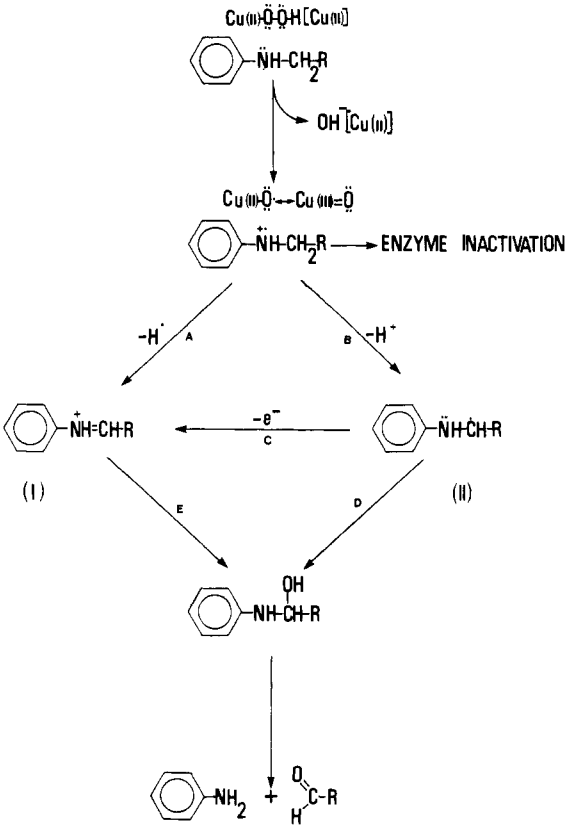
the heteroatom,[33-38] O-dealkylation of anisoles and ethoxycoumarins apparently proceeds via an α-hydroxylation mechanism.[38,39] Thus, unlike P-450, DBM is evidently unable to carry out O-dealkylation reactions via such a direct α-hydroxylation mechanism. This difference is consistent with the well-known regioselectivity of DBM for oxygenation at the benzylic position.

It has been reported that cyclopropyl benzyl ether and 1-methylcyclopropyl benzyl ether are potent suicide inhibitors for P-450, and the mechanism of inactivation is rationalized as the generation of an oxygen cation radical followed by opening of the cyclopropyl ring to generate a reactive carbon radical capable of alkylating the prosthetic heme group.[40] Such a mechanism implies that although abstraction of electrons from ether oxygen is thermodynamically unfavorable under normal conditions, certain alterations in the substrate (i.e., lack of α-hydrogen and introduction of strained ring systems) favor the generation of oxygen cation radicals by the highly electron-deficient P-450 ferryl species. In contrast, we find that the similar ether compound, 2-phenoxycyclopropylamine, is neither an inhibitor nor a substrate for DBM even under saturating oxygen concentration, although we find that the analogous carbon compound, 2-benzylcyclopropylamine, *is* a substrate for the enzyme with a k_{cat} of 4.0 s^{-1} and a K_m of 4.6 mM (unpublished results). Thus, it is clear that the effective redox potential of the activated "copper–oxygen" species of DBM is insufficient to remove an electron from the ether oxygen atom. On the other hand, nitrogen atom oxidation is comparatively a kinetically and thermodynamically facile process,[41] and N-dealkylation could readily be initiated via direct electron transfer from the benzylic nitrogen atom of the substrate.

The contrasting effect of β-Me substitution in the diamine vs. sulfide series of DBM substrates is remarkable. Thus, in going from PEDA to β-MePEDA, substrate activity becomes undetectable, while β-MePAES is an excellent DBM substrate with reactivity comparable to that of PAES itself. One obvious difference between these two series of substrates is that N-dealkylation, regardless of mechanism, must involve loss of hydrogen from the carbon adjacent to benzylic nitrogen at some point along the catalytic pathway. Thus, the decrease in the acidity of this hydrogen resulting from methyl substitution may be primarily responsible for the abolition of substrate activity in β-MePEDA; a similar explanation has been advanced for substrates of MAO.[42] An alternative explanation is that steric interference with the C–H bond cleavage step is the primary impediment to β-MePEDA turnover. We note that in the sulfur series both α- and β-Me PAES are good substrates for DBM, and in the diamine series *N*-MePEDA is also a good DBM substrate. Thus, only methyl substitution on the side chain carbon undergoing C–H bond cleavage causes an appreciable effect on turnover.

In contrast to the effect of β-Me substitution on substrate activity, no significant effect on rate of inactivation (PEDA vs. β-MePEDA) was observed, suggesting that the C–H bond cleavage step is *not* involved in the enzyme inactivation pathway.

Scheme I



In accord with this view, *N*-2-(fluoroethyl)aniline exhibits inhibitory potency similar to that of *N*-ethylaniline, despite the significant increase in kinetic acidity of the C_α proton caused by such fluoro substitution.[43] Furthermore, the deuterium isotope effect on the partition ratio measured with dideuteriated-PEDA was found to be a reflection of the isotope effect on V_{max}. Taken together, these results are in excellent agreement with the conclusion that the species responsible for enzyme inactivation is generated along the N-dealkylation pathway *prior* to the C–H bond cleavage step. Strong support for this conclusion is provided by our finding that both aniline and 5-hydroxyindole—where no abstractable C_α protons are present—are potent mechanism-based inhibitors, but not substrates, for DBM.

The fact that aniline, one of the products of DBM-catalyzed dealkylation of PEDA, is itself capable of inactivating DBM in a time- and concentration-dependent manner does not appreciably affect the results reported above for PEDA, β-MePEDA, and *N*-MePEDA. First, calculations based on turnover numbers indicate that aniline reaches a maximum of less than 10 μM concentration in typical inactivation incubations with PEDA; with *N*-MePEDA less than 5 μM *N*-methylaniline is produced; β-MePEDA does not produce any significant amount of aniline due to its extremely weak substrate activity. Control experiments have established that these small quantities of aniline or *N*-methylaniline are insufficient to produce the degree of inactivation observed with PEDA, *N*-MePEDA, and β-MePEDA. Second, the multiple addition experiments establish that the time-course of inactivation of the successive aliquots of DBM is virtually identical with that of the first aliquot in the presence of the preformed products of PEDA/DBM reaction. The concentration of aniline was estimated to be 12 μM in this system at the end of the experiment. Finally, except for PEDA and *N*-MePEDA, all of the other inhibitors investigated in this study are extremely weak substrates for DBM; therefore, it is not possible to build up appreciable amounts of aniline or aniline-like products which would be sufficient to inactivate the enzyme to any significant extent.

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While P-450-catalyzed N-dealkylations of N-substituted anilines have been studied in great detail,[41,44,45] only ring hydroxylation has been reported for primary aromatic amine substrates such as aniline.[46] In contrast, peroxidases have long been known to catalyze the oxidation of aniline and N- and ring-substituted anilines,[47-49] with the initial step being the single-electron oxidation of the nitrogen atom to generate a nitrogen-centered cation radical.[49] The major end products of these reactions arise from the head-to-tail or tail-to-tail coupling at nitrogen and/or the para carbon; species with radical character at the para position are important contributors to the resonance stabilization of the nitrogen cation radical.[49] Electrochemical oxidations of aniline[50] and N- and ring-substituted derivatives of anilines[51,52] are also well characterized and are known to be initiated by the electrochemical generation of a nitrogen cation radical followed by fast chemical radical coupling yielding both head-to-tail and tail-to-tail coupled products. These results provide important precedence for the view that benzylic nitrogen cation radicals are highly reactive species which can undergo chemical transformations readily.

On the basis of our results and these literature precedences, a general mechanism for DBM processing of aniline- and PEDA-like compounds is shown in Scheme I. While our results do not provide any information regarding the electronic nature of the copper–oxygen species along the reaction pathway, a reasonable scenario for the initial catalytic event is single-electron transfer from benzylic nitrogen to the putative Cu^{II}–O–O–H[Cu^{II}] species to yield a $[Cu^{II}—O]^+ \leftrightarrow [Cu^{III}=O]^+$ species, $OH^-[Cu^{II}]$, and the nitrogen cation radical. As discussed previously,[11] initial generation of the cation radical is fully consistent with the characteristics of DBM-catalyzed sulfoxidations, with processing of olefinic substances to give both turnover and inactivation, and with the inability of DBM to carry out O-dealkylations. As shown in the scheme, it is the nitrogen cation radical which partitions between inactivation and C–H bond cleavage at the adjacent carbon, the latter leading to N-dealkylation. Strong support for the conclusion that the inactivating species is generated *prior* to C–H bond cleavage is provided by our finding that 5-hydroxyindole and aniline, where no abstractable C_α hydrogens are present, are potent mechanism-based inhibitors for DBM, and also by the insensitivity of the inactivation potency to α-fluoro substitution, by the deuterium isotope effects on the partition ratio, and by the contrasting effect of β-methyl substitution on turnover vs. inactivation.

Both our results and literature precedence provide insight into the likely chemistry of the irreversible inactivation process at the active site of DBM. Our results with ring-deuteriated and ring-tritiated PEDA reveal that the amount of radioactivity incorporated into inactivated DBM by ring-tritiated PEDA is in agreement with that expected for covalent attachment of the para ring carbon to the protein. This is in accord with the literature findings for peroxidase and electrochemical oxidations of anilines and its ring-N-substituted derivatives, where nitrogen cation radicals readily undergo coupling reactions through the para ring carbon. We note that there is also precedence for the 5-hydroxyindole nucleus undergoing facile one-electron oxidations to generate semiquinone–imine free radicals under a variety of enzymatic and chemical conditions,[53] and these radicals are known to generate melanin-type polymers via radical coupling reactions. Uemura et al.[54] found that serotonin and 5-hydroxytryptophan irreversibly bind to microsomal proteins in the presence of NADPH and postulated the involvement of semiquinone–imine intermediates in the covalent attachment of 5-hydroxyindole moiety to these proteins. We believe that a similar reactive species is responsible for the inactivation of DBM by 5-hydroxyindole.

In P-450-catalyzed N-dealkylations, generation of the initial nitrogen cation radical is followed by either hydrogen atom ($H^\bullet$) transfer from the substrate to the Fe^{IV}=O species resulting in a Fe^{III}–OH/iminium ion pair[41,44] or transfer of a labile proton (H^+) from the α-carbon to generate a carbon-centered radical.[41] Both pathways (A and B) are shown in Scheme I, and either can be reconciled with experimental results reported to date for DBM processing of heteroatom-containing substrates. We do note, however, that, unlike P-450, DBM exhibits well-known regiospecificity for oxygenations at the benzylic position, and we have shown that DBM is unable to initiate O-dealkylation reactions via initial attack of the activated "copper–oxygen" species at the carbon adjacent to a benzylic oxygen. Whether or not this regiospecificity is relaxed after initial ganeration of the nitrogen cation radical is presently unknown; if it is not, then the second step in DBM-catalyzed N-dealkylations is most probably the abstraction of a proton from the labile C_α position of the nitrogen cation radical by a basic residue in the vicinity of the active site to produce a carbon-centered radical (pathway B).

Scheme I shows pathways for completion of the catalytic cycle from either species I or II. The imine species (I) could react with copper bound OH or solvent water to generate a carbinolamine (pathway E) which will readily undergo breakdown (either at the active site or in free solution) to the aniline and aldehyde products. The carbon-centered radical (II) could undergo either transfer of a second electron to the $[Cu^{III}=O]^+$ species to generate an imine/$[Cu^{II}—O]$ ion pair (pathway C) or oxygen rebound from the $[Cu^{III}—OH]^{2+}$ species to the α-carbon (or oxygen rebound from the $[Cu^{III}=O]^+$ species to the α-carbon followed by protonation) to produce carbinolamine (pathway D). In P-450-catalyzed N-dealkylations, an oxygen-rebound mechanism has been suggested to reconcile the incorporation of the iron-bound oxygen into the derived carbinolamine.[55-57] The results of our ^{18}O-labeling experiments demonstrate that the aldehyde oxygen of enzymatically produced 2-aminoacetaldehyde very rapidly exchanges with solvent water, a finding which is commonly observed with simple aldehydes and ketones.[30] Therefore, we were unable to trace the source of oxygen in the enzymatically produced aldehyde with ^{18}O-labeling experiments. The ^{18}O experiments did, however, provide unequivocal confirmation of the identity of 2-aminoacetaldehyde as the second product formed from DBM/PEDA turnover.

Taken together, our results provide strong support for a catalytic mechanism involving initial single-electron oxidation of benzylic-nitrogen-containing compounds by DBM. The conclusion that the nitrogen cation radical is the enzyme inactivating species is particularly intriguing since this represents, in essence, a trapping of cation radical species formed along the N-dealkylation pathway. We believe that a similar overall mechanism is functional for all DBM-catalyzed monooxygenations. Benzylic oxygenation by DBM proceeds through the unified intermediacy of a species with cationic and/or radical character when either methylene, olefin, carbinol, sulfur, or nitrogen is in the benzylic position. Oxygen transfer from copper to this species occurs readily in the case of carbon; similarly, the strong tendency of the sulfur cation radical toward sulfoxidation[40,41] produces solely the sulfoxide and not the S-dealkylated product. For nitrogen, N-dealkylation of the cation radical species is a favored process and occurs readily.[40,41]

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The newly discovered N-dealkylation activity of DBM is a potentially valuable tool for the understanding of the chemistry of DBM catalysis at the molecular level. Moreover, we note that this N-dealkylation activity is directly analogous to the peptidylglycine amidating monooxygenase reaction which has been recently shown to be catalyzed by an oxygenase very similar to DBM in its subcellular location and requirement for copper and ascorbate.[58] Furthermore, we note that in view of the central role of DBM in adrenergic neuronal function, these substrate analogues and inhibitors are valuable in designing pharmacologically interesting compounds that might be important for the modulation of dopamine/norepinephrine levels in vivo.[59-62]

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A Kinetic Rationale for the Inefficiency of 5-Iminodaunomycin as a Redox Catalyst

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Abstract: The redox chemistry of 5-iminodaunomycin (**1**) is compared with the redox chemistry of daunomycin (**2**) to establish a possible rationale for the inefficiency of 5-iminodaunomycin as an in vivo redox catalyst for the production of reactive oxygen species. Anaerobic reduction of **1** with sodium dithionite in methanol solvent gives 5-imino-7-deoxydaunomycinone (**3**) most likely via the intermediate quinone methide **6**. The slow step is tautomerization of **6** to **3**, a pseudo-first-order process with a rate constant of 2.2×10^{-2} s^{-1} at 25 °C. Anaerobic reduction of **3** with sodium dithionite in methanol solvent gives 2-acetyl-2,11-dihydroxy-7-methoxy-1,2,3,4-tetrahydro-5,12-naphthacenedione (**4**) most likely via the intermediate naphthacenone **8**. The slow step, proposed to be loss of ammonia, occurs with a rate constant of 4.0×10^{-2} s^{-1} at 25 °C. Even traces of molecular oxygen interfere with these two reductions, hence **1** and **3** should be efficient catalysts for the production of reactive oxygen species. Reduction of **4** with sodium dithionite in anaerobic methanol or methanol purged with 2% oxygen rapidly gives 8-acetyl-1-methoxy-7,9,10,12-tetrahydro-6,8,11-trihydroxy-5(8*H*)-naphthacenone (**9**). Naphthacenone **9** is slowly oxidized back to **4** by molecular oxygen with a pseudo-first-order rate constant of 3.3×10^{-4} s^{-1}. Although reduction of daunomycin by sodium dithionite in parallel fashion gives 7-deoxydaunomycinone (**5**), reduction of 7-deoxydaunomycinone does not rapidly give **4** but slowly gives 2-acetyl-1,2,3,4,4a,12a-hexahydro-2,6,11-trihydroxy-7-methoxy-5,12-naphthacenedione (**11**) with a rate constant of 1.5×10^{-4} s^{-1}. Naphthacenedione **11** is reasonably insensitive to molecular oxygen. Upon standing in the presence of oxygen very slow elimination to give **4** and oxidation to give **5** occur. Consequently, a possible explanation for the inefficiency of 5-iminodaunomycin for catalyzing in vivo the reduction of molecular oxygen is the facile formation of naphthacenedione **4** which is an inefficient catalyst. The inefficiency arises because the hydroquinone of **4** tautomerizes to naphthacenone **9** in preference to reduction of molecular oxygen.

5-Iminodaunomycin (**1**) is a semisynthetic derivative of the pharmacologically important anti-tumor drug daunomycin (**2**) in which the C-ring quinone has been transformed regiospecifically into an iminoquinone.[1] It has attracted significant attention because it shows less cardiotoxicity than daunomycin while retaining significant anti-tumor activity.[2] The lower cardiotoxicity of **1** has been rationalized in terms of a diminished potential for catalytic production of reactive oxygen species which presumably attack heart cell membrane lipids.[3-5] Daunomycin and adriamycin efficiently catalyze the production of reactive oxygen species in the presence of either a one- or two-electron reducing agent and molecular oxygen;[6-10] this process is sometimes referred to as radical recycling. 5-Iminodaunomycin has been recently described in an excellent review of the anthracyclines as a redox-incapacitated anthracycline, one which is both more difficult to reduce and/or reoxidize and, hence, cannot catalyze efficiently the reduction of molecular oxygen.[11]

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