# Rapid Freeze- and Chemical-Quench Studies of Dopamine β-Monooxygenase: Comparison of Pre-Steady-State and Steady-State Parameters†

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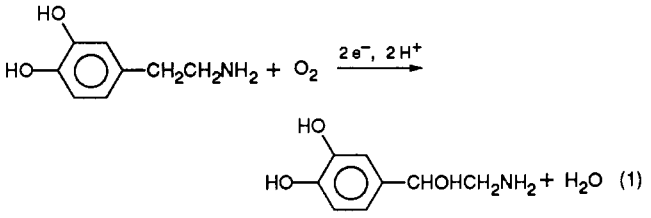
ABSTRACT: The copper-containing enzyme dopamine β-monooxygenase has been studied with regard to pre-steady-state kinetics of tyramine hydroxylation and reduction of enzyme-bound $Cu^{2+}$ by chemical- and freeze-quench EPR techniques. The bulk of the enzyme-bound copper (~70%) is reduced in a single-exponential process with a limiting rate constant of 250 $s^{-1}$, $K_m$ = 0.9 mM, consistent with participation of both copper ions in the redox events of catalysis. The remaining copper is reduced much more slowly ($k \sim 2\ s^{-1}$) or not at all, attributed to a distribution of copper into inhibitory binding sites and the presence of some inactive enzyme. Knowledge of the $Cu^{2+}$ reduction rate, together with rate constants calculated from steady-state isotope effects [Miller, S. M., & Klinman, J. P. (1985) *Biochemistry 24*, 2114-2127], has allowed prediction of pre-steady-state product formation transients. Measurement of these transients under conditions of excess ascorbate shows close agreement with prediction, supporting the validity of individual rate constants obtained from steady-state data. Kinetic modeling shows further that the predominant steady-state enzyme form is the enzyme–product complex (E–P), which is expected to show a correspondingly large (~70% of total copper) EPR signal for bound $Cu^{2+}$. Surprisingly, the steady state is characterized by a low (19% of total copper) EPR signal. This lack of correlation between the anticipated and observed steady-state EPR signal suggests either antiferromagnetic coupling in binuclear copper centers or reduction of $Cu^{2+}$ in this enzyme form by ascorbic acid. The latter possibility is favored since single-turnover studies rule out significant coupling between copper centers in E–P [Brenner, M. C., & Klinman, J. P. (1989) *Biochemistry* (following paper in this issue)].

Dopamine β-monooxygenase (DβM, EC 1.14.17.1) catalyzes the hydroxylation of dopamine to norepinephrine, coupled to a two-electron oxidation of electron donors such as ascorbic acid:



(1)

The enzyme has been studied extensively with regard to copper cofactor requirements and kinetic and chemical mechanism [for recent reviews, see Stewart and Klinman (1988), Ljones and Skotland (1984), and Villafranca (1981)]. Some of the major conclusions relevant to the studies addressed in this and the following paper include (1) a requirement for two copper ions per subunit in the expression of full catalytic activity (Klinman et al., 1984; Ash et al., 1984), although the two bound copper ions appear spectroscopically similar and show no indication of significant antiferromagnetic coupling (Villafranca, 1981; Brenner, 1988; McCracken et al., 1988); (2) the interaction of exogenous electron donors with a different (presumably oxidized) form of enzyme from that which binds phenethylamine and $O_2$ substrates (Goldstein et al., 1968; Fitzpatrick et al., 1986; Stewart & Klinman, 1988), suggesting that enzyme can be loaded with a full complement of reducing equivalents prior to substrate binding; and (3) the predominance of the enzyme–product complex in the steady state due to a much slower release of bound product than C–H bond cleavage (Miller & Klinman, 1985).

The detailed role of copper in catalysis remains a central question regarding the mechanism of DβM. It has been proposed that both bound copper ions serve as vehicles for electron transfer between the exogenous reductant and $O_2$ (Klinman et al., 1984), although the likelihood of involvement of two copper ions per subunit in this process has been disputed (Syvertsen et al., 1986). In an effort to resolve this issue, we undertook a series of rapid chemical and freeze–quench EPR[1] experiments involving reduction of enzyme-bound copper and correlation of product formation with copper oxidation state. In this paper we show first that *both* cupric ions per subunit can be reduced by ascorbate at a rate that is consistent with catalysis. Measurement of pre-steady-state tyramine hydroxylation in the presence of excess ascorbate leads to product formation transients which can be accurately modeled by rate constants obtained from steady-state isotope effects (Miller & Klinman, 1985), supporting the validity of this approach to obtaining rate constants for the elementary steps of a minimal mechanism. When the transient for enzyme-bound $Cu^{2+}$ was monitored by EPR under conditions similar to those of the analysis of product transients, the signal anticipated for $Cu^{2+}$ in the enzyme–product complex failed to reappear. This unexpected observation, in conjunction with single-turnover data described in the accompanying paper (Brenner & Klin-

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[1] Abbreviations: EPR, electron paramagnetic resonance; HPLC, high-pressure liquid chromatography; $KP_i$, potassium phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Trizma-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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man, 1989), leads to a new model for the interaction of DβM with reductants.

## Materials and Methods

*Materials.* Ascorbic acid, tyramine hydrochloride, isopentane, and benzylidenemalononitrile were from Aldrich; potassium phosphate, $CuSO_4$, and certified atomic absorption copper standards were from Fisher Scientific; dopamine hydrochloride, disodium fumarate, MES buffer, 1-heptanesulfonic acid sodium salt, octopamine hydrochloride, Trizma-HCl, and horse heart metmyoglobin were from Sigma Chemical Co.; catalase was from Boehringer Mannheim; potassium ferricyanide was from Allied Chemical. Concanavalin A–Sepharose 4B, Sephacryl S-300, and Sephadex G-25 were from Pharmacia Fine Chemicals, Inc.; Ultrogel AcA 34 was from LKB (now available from IBF Biotechnics); DE-52 ion-exchange resin was from Whatman. HPLC columns, Teflon tubing, Kel-F 1/4-28 nuts, and three-way Hamilton valves (No. 86727) were from Rainin Instrument Co.; TEFZEL ferrules were from E & K Scientific; EPR tubes were prepared from commercial-grade 3-mm i.d. × 4-mm o.d. quartz tubing from Heraus-Amersil, or precision-bore tubing was obtained from Wilmad Glass Co.

*Isolation of Soluble Dopamine β-Monooxygenase.* DβM was isolated by one of two methods similar to the one described by Scott et al. (1988). In the first procedure, whole bovine adrenal medullae (~1000 g) were homogenized in four batches in 5 mM $KP_i$, pH 6.5, containing 30 μg/mL catalase (4 L total). Following centrifugation at 100000*g* (20 min) the supernatant was filtered through glass wool and loaded onto a 50-mL column of concanavalin A–Sepharose 4B at a flow rate of ~200 mL/h. After being loaded, the column was washed overnight at the same flow rate with 50 mM $KP_i$–0.2 M KCl, pH 6.5 (wash buffer). The protein was eluted with a linear 0–10% gradient of methyl α-D-mannopyranoside in the wash buffer. Eluted fractions having significant absorbance at 280 nm were pooled and concentrated by precipitation with $(NH_4)_2SO_4$, as described by Scott et al. (1988), followed by passage through a column of Sephadex G-25 equilibrated with 5 mM $KP_i$, pH 6.5. The desalted protein was loaded onto a column of DE-52 and eluted as described by Klinman and Krueger (1982). The eluted protein, concentrated to ~5 mL, was loaded at a flow rate of ~7 mL/h onto a size-exclusion column of Ultrogel AcA 34 or Sephacryl S-300 (2.5 cm × 100 cm) equilibrated with wash buffer. The main peak of absorbance was pooled and concentrated in an Amicon concentrator (PM-10 membrane). The protein was passed through a column of Sephadex G-25 equilibrated in 5 mM $KP_i$, pH 6.5, or 10 mM MES, pH 6.0, reconcentrated to 5–8 mg/mL, centrifuged to remove precipitated material, and stored in 200-μL aliquots at −70 °C. The final protein concentration was determined by absorbance [$\epsilon_{280}$ = 1.24 (cm·mg/mL)$^{-1}$ (Skotland & Ljones, 1977)]. This method yields ~70 mg of DβM/kg of adrenal medulla.

An alternative method of isolation involved partial purification of chromaffin granules as described by Klinman et al. (1980), omitting the final sucrose density gradient centrifugation step. The crude vesicles were stored at −70 °C and later thawed, lysed, centrifuged, and chromatographed by the procedure described above. This procedure gave enzyme with similar specific activities and pre-steady-state behavior as enzyme from the whole medullae method, but with half the yield.

*Measurement of Enzyme Activity.* The steady-state rate of oxygen consumption was measured with a polarographic oxygen electrode (Yellow Springs Instrument Model 53 biological oxygen monitor). Reaction conditions were as follows: 5–10 μg/mL DβM, 1 mM dopamine hydrochloride, 0.21 mM $O_2$ (air saturation), 10 mM ascorbic acid, 10 mM disodium fumarate, 10 μg/mL catalase, and 100 mM $KP_i$, pH 6.0, $T$ = 35 °C. Specific activities of isolated enzyme were generally within 10% of 6.0 μmol of $O_2$ consumed per minute per milligram of DβM.

*Total Copper and $Cu^{1+}$ Content of Isolated DβM.* The purified enzyme was assayed for total copper content as described by Klinman et al. (1984). The percentage of $Cu^{1+}$ in each enzyme preparation was determined by observing the magnitude of the $Cu^{2+}$ EPR signal before and after enzyme oxidation with potassium ferricyanide. Typically, the EPR spectrum of DβM as isolated was recorded followed by addition of a 5-fold excess of potassium ferricyanide over enzyme-bound copper. The EPR signal was recorded and another aliquot of ferricyanide added to oxidize any remaining $Cu^{1+}$. A 10-fold excess of ferricyanide over enzyme-bound copper was always sufficient to give a maximal $Cu^{2+}$ EPR signal.

*Electron Paramagnetic Measurements.* EPR measurements of the enzyme's $Cu^{2+}$ content were made with a JEOL-ME-type EPR spectrometer operating at 9.2 GHz with 100-kHz modulation of the magnetic field. Microwave power was precisely set with the use of a power meter (Hewlett-Packard Model 432A) and an absorptive PIN-diode (General Microwave Corp. Model D-1958). Tuning of the EPR spectrometer was accomplished by critically coupling the loaded cavity with the microwave source by minimizing the return power from the cavity. Signal averaging and integration of spectra were performed with a PDP-8/L digital computer with an AXO8 analog-to-digital converter. A quartz dewar assembly permitted sample cooling to liquid helium temperatures. Samples were placed in a cylindrical-mode ($TE_{011}$) EPR cavity (JEOL UCX-2), with a loaded quality factor ($Q_L$) of ~5000. Unless stated otherwise, EPR settings were as follows: 5-mW microwave power, 20-G modulation width, 2970 ± 500 G field sweep, 57 s/scan × four scans, 0.3-s time constant, and $T \cong$ 16 K. During EPR measurements of freeze–quenched samples the EPR tubes were capped with a 5-mL disposable syringe attached by latex tubing. This arrangement prevented condensation of air in the EPR tube and also allowed for gas expansion in the tube during handling.

Comparison of one EPR signal to another involved corrections for different EPR gain settings as well as small (<15%) variations in sample size. To correct for variations in the height of the frozen samples, a calibration plot of EPR signal versus sample height was prepared with a $Cu^{2+}$-EDTA standard. Small corrections (<2%) were also made for variations in EPR tube diameter.

*Rapid-Mixing Apparatus.* All rapid-mixing experiments on the millisecond time scale were carried out with a modified Update Instruments System 1000 chemical/freeze-quench apparatus. A syringe block with thermostated cavity and water jackets for syringe connection hoses were constructed to assure accurate temperature control of reacting solutions without requiring submersion of the syringes and hoses as intended by the original design. The original syringe plungers were replaced by aluminum rods ($^1/_4$-in. diameter) with permanently attached Teflon tips machined to give a tight fit in the syringe barrels (2.5 mL 0.337-cm$^2$ cross-sectional area) at 25 or 35 °C. The original nylon hoses with stainless 1/4-28 connectors were replaced by Teflon hoses (0.5- or 0.8-mm i.d. × $^1/_{16}$-in. o.d.), TEFZEL ferrules, and Kel-F 1/4-28 nuts which were machined to be used with TEFZEL ferrules, giving excellent

chemical resistance of the entire system. The original zinc mixing grids in the Whiskind mixer deteriorated rapidly with use and were replaced by gold grids. Valves were also installed to facilitate washing and drying of the flow system (see Rapid Acid-Quench Experiments with DβM).

*Freeze-Quenching Procedures.* The equipment required for freeze quenching was patterned after that described previously (Ballou, 1971; Bray, 1961; Bray & Petterson, 1961; Bray et al., 1964). The temperature of the liquid isopentane, monitored with an electronic thermometer from Omega Engineering, was maintained at −135 ± 5 °C. In a typical experiment, a combined volume of 0.33 mL from two syringes was pushed at a velocity of 2 cm/s through a reaction hose (referred to as aging hose) and spray nozzle (0.008 in. diameter exit hole) into an isopentane reservoir with EPR tube attached. These conditions gave crystals in the reservoir which could be packed within 5 or 10 min to produce a sample 25 mm in height at the bottom of the EPR tube. The excess isopentane was aspirated away from the packed samples, and the tubes were stored in liquid $N_2$ for EPR measurements at a later time.

*Determination of Instrument Dead Time.* The base hydrolysis of benzylidenemalononitrile, a reaction with good first-order kinetic behavior at high pH (Bernasconi et al., 1984), was employed as a standard reaction. The benzylidenemalononitrile stock was prepared by dissolving 5 mg of benzylidenemalononitrile in 2 mL of DMSO and diluting 0.6–1.2 mL of this solution into 100 mL of a 0.5 M KCl solution containing 1 mM HCl. Zero time points were determined by rapid mixing of this stock from an Update Instruments syringe with 0.5 M KCl from a second syringe and "quenching" through a second mixer with 0.4 M KCl and 40 mM HCl from a third syringe. For the remaining time points the benzylidenemalononitrile was rapidly mixed with 0.4 M KOH plus 0.1 M KCl and the reaction stopped by quenching with 0.44 M HCl from a third syringe. An infinity point was determined by hand mixing the reactants for ~15 s and quenching with 0.44 M HCl, giving an $OD_{309}$ of between 15% and 23% of the time zero value. This absorbance was subtracted from all measurements before semilogarithmic plots were made. Reaction time was a function of flow velocity and the volume of the aging hose. An acid delay hose with half the volume of the aging hose prevented premature backflow of acid into the aging hose. Semilog plots of quenched sample absorbance vs nominal reaction time yielded a pseudo-first-order rate constant of 24.5 $s^{-1}$ ($[OH^-]$ = 0.2 M, $T$ = 25 °C) for ram speeds greater than or equal to 2.0 cm/s (1.35 mL/s flow rate through the aging hose), in agreement with previously reported values. The "dead time" (4 ± 2 ms) was determined as the number of milliseconds which, when added to the nominal reaction times, gave intersection of the absorbance at time zero with the linear fit to the remaining time points.

The reaction of metmyoglobin with azide ion was employed as a standard reaction in evaluating dead times for the freeze-quench method (Ballou, 1971). Approximately 100 mg of horse heart metmyoglobin was dissolved in 6 mL of buffer (0.02 M Trizma-HCl, 0.1 M $KNO_3$, adjusted to pH 7.8 with KOH) and dialyzed against 500 mL of the same buffer overnight at 4 °C. The dialyzed protein was centrifuged to remove any precipitated material, and this solution was shot against buffer to obtain zero time points, or against 25 mM sodium azide in buffer. The intensity of the EPR signal at $g \cong 5$ was used as a measure of unreacted myoglobin. The EPR settings were as follows: microwave power = 1.0 mW, $T \cong 15$ K, modulation width = 20 G, modulation frequency = 100 kHz, and sweep = 1100 ± 250 G. The dead time (4 ± 3 ms) was determined graphically as described above.

*Freeze-Quench Experiments with DβM.* For comparison of freeze-quenched samples with non-freeze-quenched standards, a packing factor for the effect of freeze quenching on the EPR signal magnitude was determined. Samples of $Cu^{2+}$-EDTA were prepared by freeze quenching and compared with frozen aqueous $Cu^{2+}$-EDTA standards. The second integrals of the freeze-quenched samples were 57 ± 10% as large as those of the frozen aqueous standards; i.e., the packing factor was 0.57. In practice, the EPR spectra of freeze-quenched samples of 15 μM DβM with 2 $Cu^{2+}$/subunit are very noisy at the optimum EPR settings (see Figure 1), making quantitation by double integration difficult. However, for seven freeze-quench experiments employing four different preparations of enzyme, the calculated $Cu^{2+}$ content from doubly integrated spectra (corrected for the packing factor) averaged 11% below the expected value. The two worst cases gave integrated DβM signals corresponding to a $Cu^{2+}$ content 23% below and 10% above the expected values. The 11% difference in the average probably reflects error in the packing factor or some denaturation of protein and loss of bound $Cu^{2+}$ during freeze quenching. Therefore, the $Cu^{2+}$ content of the enzyme stock determined by atomic absorption (Klinman et al., 1984) was taken to be unchanged by the freeze-quenching process, and the absolute concentrations of E–$Cu^{2+}$ in freeze-quenched samples containing reaction components were calculated relative to the signals from enzyme freeze quenched with buffer alone.

*Removal of Isopentane from Freeze-Quenched DβM.* In order to establish the activity of DβM after it had undergone the freeze-quenching process, freeze-quenched samples of enzyme in phosphate buffer were immersed in vacuo (mechanical pump) in a dry ice–acetone bath at −69 °C for 3–10 h to remove liquid isopentane. The remaining ice crystals were then thawed at room temperature. Despite extensive evacuation of the semifrozen samples, a thin layer of isopentane was visible on top of the thawed protein solution, and denatured protein was apparent at the interface of the two phases. Proteinaceous foam and traces of isopentane were aspirated away, and the remaining solution was filtered through 0.45-μm filters (Rainin Micropartition System). The protein concentration in the filtrate was determined by the method of Bradford (Bio-Rad protein assay) by comparison with DβM standards. Enzyme activity was determined as described above.

*Analysis of DβM Reduction Kinetics.* Kinetic data for the disappearance of the enzyme's $Cu^{2+}$ EPR signal due to reaction with ascorbate were fit to a sum of two exponentials:

$$ECu^{2+} = Ae^{-kt} + Be^{-jt} + C \quad (2)$$

where $A$ and $B$ are the amplitudes of fast and slow phases with rate constants $k$ and $j$, respectively, and $C$ is the amount of nonreducible copper. Nonlinear least-squares regression analysis was done on an IBM-PC/AT using the NONLIN module of the statistical package SYSTAT from SYSTAT, Inc., Evanston, IL.

*Rapid Acid-Quench Experiments with DβM.* In a typical experiment, the enzyme stock contained 20 μM DβM subunits, enough $CuSO_4$ to give a total Cu/subunit stoichiometry of 2:1, 10 mM fumarate, 100 mM $KP_i$, and 100 mM KCl. The substrate stock contained 2–60 mM tyramine hydrochloride, 20 mM ascorbic acid, 10 mM fumarate, and enough KCl to give a total chloride concentration of 100 mM. For each time point, approximately 0.15 mL of these stocks was dispensed at a 2 cm/s ram speed and the reaction stopped by mixing through a second mixer with 0.75 M $HClO_4$ from a third

syringe. The quenched samples were immediately cooled to −70 °C and stored for subsequent analysis of octopamine by HPLC.

In our hands it was not possible to obtain reproducible results with the rapid-mixing flow system as provided by Update Instruments because of small amounts of back-mixing of enzyme and substrate into the connector hoses between the first mixer and the syringes. Because these experiments were done under catalytic conditions with substrate in large excess over enzyme, a few tenths of a microliter of such contamination between shots was sufficient to create false, irreproducible "bursts" of product formation in pre-steady-state time courses (Murray & Klinman, 1985). To circumvent this problem, three-way Hamilton valves were installed between the first mixer and the hoses leading from the enzyme and substrate syringes. An additional acid delay hose was installed between the second mixer and the acid hose to account for the dead volume introduced by the valves. After each shot, the mixers and aging hose were flushed through the three-way valves with water and then dried with compressed air. The acid delay hoses were also rinsed and dried between shots. There was no leakage around the valves as long as proper fittings were employed.

*Measurement of Octopamine Formation by HPLC.* Octopamine was detected at 224 nm following HPLC separation from the other reaction components using a 30 mm × 4.6 mm, 5-μm RP-18 precolumn and a water-jacketed 0.5 cm × 25 cm Altex C-18 Ultrasphere reverse-phase column maintained at 30–45 °C. The mobile phase was 5 mM acetic acid (adjusted to pH 5.8 with aqueous $NH_3$) plus 20–70 μM heptanesulfonic acid and 4–6.5% (v/v) methanol at a flow rate of 1 mL/min. The acidic quenched samples were centrifuged to pellet-precipitated material, and 50 μL was injected without neutralization. A standard curve with mock quenched reaction mixtures (based on integrated peak area by use of a Shimadzu Chromatopac C-R3A data processor) was always linear through the range of interest.

*Computer Simulation of Product Formation Transients.* In order to model the pre-steady-state kinetics of enzyme catalysis, it was necessary to integrate the differential equations pertaining to the mechanism in question. Rather than attempt an analytical solution of these equations, a numerical approach was used based on the Runge–Kutta formula (Boyce & DiPrima, 1977), using a program in BASIC described by Scraton (1984). Once supplied with a set of rate constants and the initial concentration of enzyme, the program calculates the concentrations of each enzyme species and free product over any desired time interval. Accurate results are only obtained when the program divides the time interval into small enough step sizes (Boyce & DiPrima, 1984), a parameter fixed by the user. To check for accuracy of the numerical integration, decreasingly smaller step sizes were employed until the calculated results did not change by more than 0.1%.

## Results and Discussion

*EPR Spectra and Activity of Freeze-Quenched DβM.* The first-derivative EPR spectrum of freeze-quenched DβM with two cupric ions per subunit differs from that of DβM which was slowly frozen in an EPR tube by the presence of a small absorption of variable intensity at $g = 2.00$ (see Figure 1B). The position and *apparent* width of the peak (~14 G) is similar to those of the $g = 2.00$ peak observed in freeze-quenched samples of DβM mixed with ascorbate (Figure 1C), which presumably arises from the semidehydroascorbate radical, $g = 2.00518$ (Bielski, 1982; Skotland & Ljones, 1980). The actual width of the ascorbate radical signal is less than

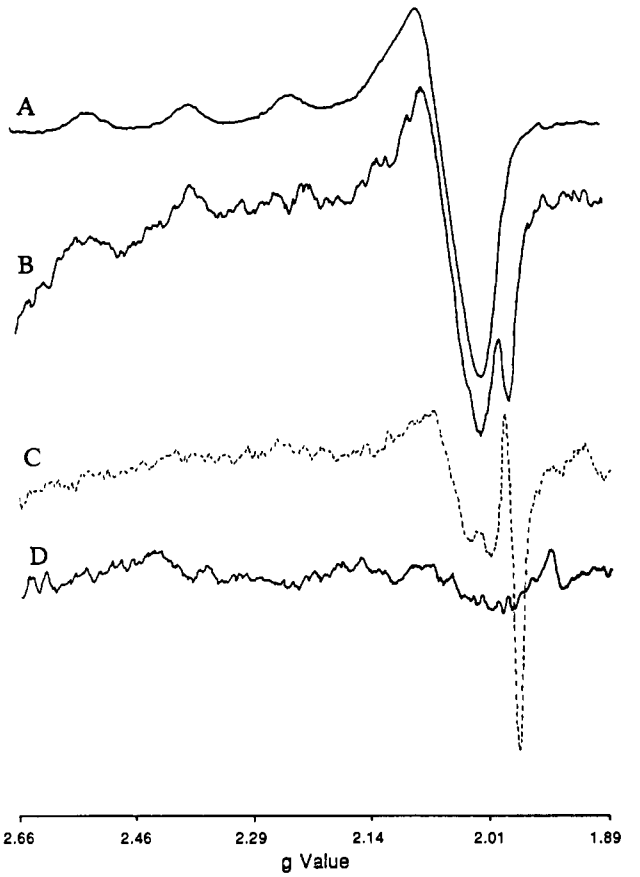



FIGURE 1: EPR spectra of freeze-quenched DβM. Unless stated otherwise, EPR settings were as follows: 5-mW microwave power, 20-G modulation width, 2970 ± 500 G field sweep, 57 s/scan × four scans, 0.3-s time constant, and $T \cong 16$ K. EPR gain was identical throughout. (A) Non-freeze-quenched 57.9 μM DβM subunits in 5 mM $KP_i$, pH 6.5, with 2 $Cu^{2+}$/subunit: microwave power = 0.2 mW, modulation width = 10 G, and $T = 14.7$ K. (B) Freeze-quenched 15 μM DβM subunits with two coppers per subunit in 25 mM potassium phosphate with 10 mM disodium fumarate and 5 mM chloride, pH 6.0. (C) Freeze-quenched DβM as in (B), after reaction with 2.0 mM ascorbic acid for 23 ms. (D) The cavity signal was obtained with an EPR tube containing distilled water.

2 G (Bielski, 1982), but in order to maximize signal to noise in the $Cu^{2+}$ region of these spectra, a modulation width of 20 G was employed, leading to severe broadening due to overmodulation (Randolph, 1972). The $g = 2.00$ absorption (Figure 1B) appears to be specific to freeze-quenched enzyme since it was absent from spectra of freeze-quenched solutions of $Cu^{2+}$-EDTA and phosphate buffer (not shown). The size, shape, and position of the peak suggest a very small concentration of organic radical (estimated to be less than 0.2% of the total area under the first-derivative spectrum) which is either revealed or formed in the enzyme by the freeze-quenching process. The nature of this absorption was not investigated further. The small absorption at $g = 2.03$ (Figure 1C) was not always present in freeze-quenched samples of DβM and was occasionally observed in spectra of distilled water as well as in freeze-quenched solutions of phosphate buffer, suggesting that it is not intrinsic to the enzyme.

To establish the activity of the enzyme after freeze quenching, nine freeze-quenched samples of DβM in phosphate buffer were thawed and analyzed as described under Materials and Methods. In nine samples an average of 41 ± 8% of the DβM protein was precipitated after the thawing procedure. Much of this precipitation may have been due to traces of isopentane that were not removable by evacuation. Remarkably, the protein which remained in solution through the

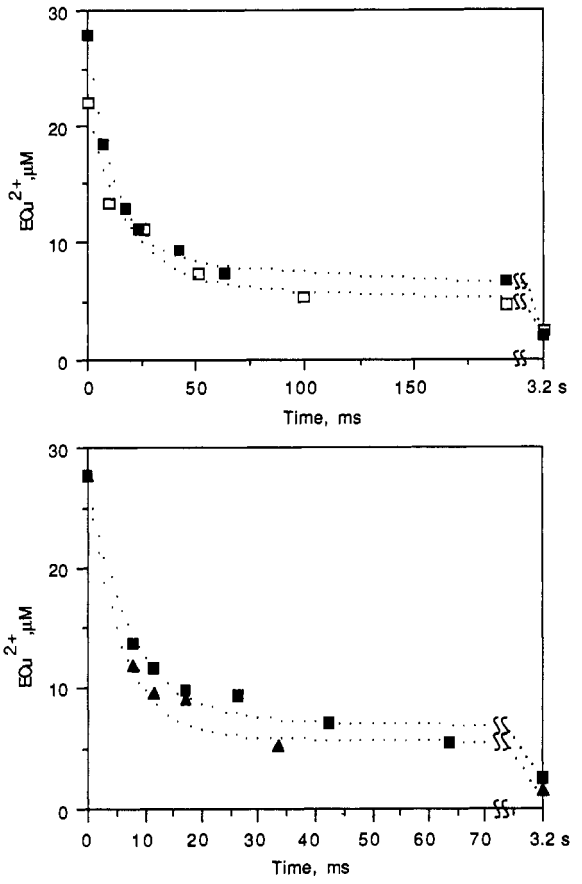



FIGURE 2: Reduction of enzyme-bound $Cu^{2+}$ by ascorbate. The disappearance of the $Cu^{2+}$ EPR signal in freeze-quenched samples of 15 μM DβM (containing 30 μM $Cu_{total}$) mixed with ascorbate for the indicated times at 35 °C is shown. Ascorbate concentrations after mixing were as follows: (top panel) (□) 0.2 mM (12 μM DβM and 24 μM $Cu_{total}$ for this ascorbate concentration) and (■), 0.5 mM; (bottom panel) (■) 1 mM and (▲) 2.0 mM. Dotted curves are simulations based on eq 2 in the text (see Table I for best-fit parameters).

Table I: Kinetic Parameters of DβM Reduction[a]

| ascorbate (mM) | $k$ ($s^{-1}$) | $A$ (%) | $B$ (%) | $C$ (%) | $j$ ($s^{-1}$) |
|---|---|---|---|---|---|
| 0.2 | 56 ± 14 | 70 | 18 | 12 | 2 |
| 0.5 | 83 ± 17 | 68 | 24 | 8 | 2 |
| 1.0[b] | 135 ± 25 | 73 | 18 | 9 | 2 |
| 2.0 | 176 ± 40 | 78 | 17 | 5 | 2 |

[a] Parameters were determined by nonlinear least-squares fitting of experimental data to eq 2 in the text. The values of $C$ are the percentage of nonreducible copper in each experiment. The slow phase rate constants, $j$, were fixed in the regression analysis to the value shown. Uncertainties in the amplitudes $A$ and $B$ were less than 8% and 30%, respectively, of the values listed. [b] In a previous study the rate of reduction by 0.9 mM ascorbate of $Cu^{2+}$ in enzyme containing only 1.0 mol of copper/mol of subunits was ~20 $s^{-1}$ at 22 °C, pH 6.8 (Skotland et al., 1980).

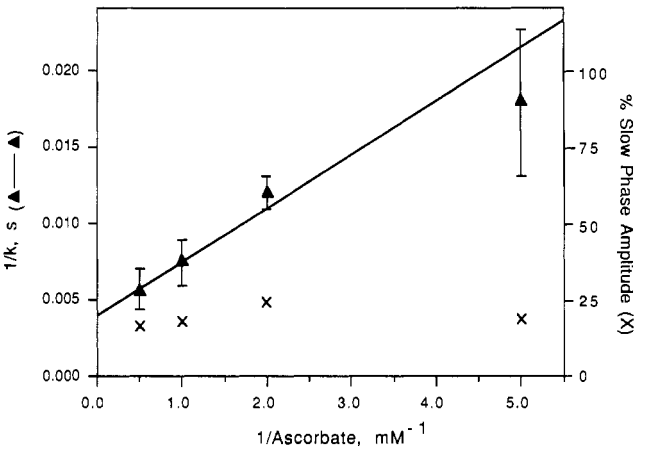


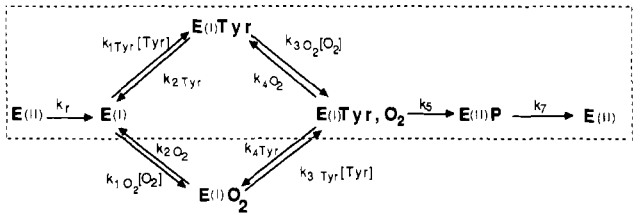

FIGURE 3: Limiting rate of enzyme reduction by ascorbic acid. The reciprocals of the fast-phase rate constants, $k$ (Table I), are plotted vs 1/[ascorbate] (▲). The limiting rate at saturating ascorbate is 250 ± 30 $s^{-1}$, and the half-maximal rate occurs at 0.9 ± 0.2 mM ascorbate. The percentage of copper reduced in a slow phase [$B$ (%) in Table I] is also shown (×).

thawing process retained 100 ± 20% of its original enzymatic activity.

*Reduction of Enzyme-Bound Copper.* Our preliminary experiments, conducted with a syringe ram speed of 1.25 cm/s, indicated a rapid phase of reduction for about 70–80% of the enzyme-bound copper and a very slow phase of reduction corresponding to ~20% of the total copper (data not shown). Ascorbate concentrations of 0.2, 0.25, and 0.33 mM gave slow phase rate constants of 2, 1, and 0.8 $s^{-1}$, respectively, on the basis of the nonlinear regression fit to eq 2 (see Materials and Methods). Subsequently, measurements of instrument dead time suggested that mixing might be incomplete with a 1.25 cm/s ram speed, and experiments were later repeated at 2 cm/s. The results of these rapid-freeze quench reduction experiments at 2 cm/s ram speed with DβM containing two copper ions per subunit and ascorbic acid concentrations from 0.2 to 2.0 mM are shown in Figure 2. As seen by others with enzyme containing only one copper per subunit (Skotland et al., 1980), a small amount (8–10%) of the enzyme-bound copper is not reduced by ascorbate under these conditions. The reduction data shown in Figure 2 were fit to eq 2 (results in Table I), although the slow phase rate constant was fixed at 2 $s^{-1}$ because there were insufficient data points at long reaction times to allow a computer estimation of this parameter. The fixed value of $j$ is not critical since a 3-fold change in the assumed value of $j$ led to only a 5–10% change in the computer fit value of the fast-phase rate constant. The dashed lines in Figure 2 correspond to the parameters listed in Table I for each ascorbate concentration. A replot of the reduction data is given in Figure 3, where the solid line is a nonlinear least-squares fit calculated with the program HYPER (Cleland, 1979). A limiting reduction rate of 250 ± 30 $s^{-1}$ and an apparent $K_m$ of 0.9 ± 0.2 mM were obtained. Addition of 100 mM chloride ion to the enzyme and ascorbate solutions did not have a significant effect on the rate of enzyme reduction.

These reduction data show that the bulk of the enzyme-bound copper can be reduced by ascorbate in a single-exponential process, consistent with the involvement of both copper ions in the redox events of catalysis as proposed by Klinman et al. (1984). The finding that 20% of the EPR-detectable enzyme-bound copper is reduced at a rate of only 2 $s^{-1}$ and that 10% of the copper is not reduced at all requires explanation, however. Contamination of enzyme preparations by other proteins is unlikely since SDS–polyacrylamide gels of purified DβM are routinely free of significant levels of extraneous, Coomassie-blue staining bands. Although proteins such as chromagranin A could comigrate with DβM in these gels (Apps et al., 1985), chromagranins do not bind to concanavalin A and are expected to be lost early in the DβM preparation. In contrast, DβM inactivated by hydrogen peroxide has been shown to copurify with active enzyme (Colombo et al., 1987). While this is unlikely to be a major problem in our preparations due to the presence of added catalase, some inactivation by hydrogen peroxide may be unavoidable and cannot be excluded as the source of nonreducible copper.

Scheme I: Minimal Kinetic Mechanism for Tyramine Hydroxylation by Dopamine β-Monooxygenase [Adapted from Ahn and Klinman (1983)][a]




[a] The expected oxidation state of both copper ions is indicated parenthetically as (I) or (II).

A more likely source of the biphasicity in enzyme reduction transients originates in the distribution of copper into inhibitory copper binding sites. As shown in our initial study of DβM activation by copper, activity curves reflect the presence of a third, lower affinity inhibitory copper site, in addition to two catalytic copper sites (Klinman et al., 1984). A consequence of the competition between inhibitory and catalytic copper binding is a reduction in the concentration of active enzyme at a stoichiometry of two coppers per subunit to only 80% of total enzyme concentration. In this earlier study, curve fitting led to estimates of p$K$ values of 8.0 and 5.3–5.9 for the binding of catalytic and inhibitory reduced $Cu^{1+}$, respectively. In the present investigation of enzyme reduction, the resting form of enzyme contains $Cu^{2+}$, and so the initial distribution of copper into catalytic and inhibitory sites will reflect binding of the cupric ion. In a study by Syvertsen et al. (1986) using a copper-sensitive electrode, only one tight binding site per subunit for $Cu^{2+}$ was reported with a p$K$ of approximately 11. More recently, Backburn has observed p$K$ values of 8.9 and 7.0 for the first and second $Cu^{2+}$ bound per subunit.[2] Using the latter data for the binding of catalytic copper and a p$K$ of 5.9 for the binding of inhibitory copper, at a copper per subunit stoichiometry of 2:1, we estimate that 11% of the total copper is in an inhibitory site, with 21% of total enzyme containing copper in this site.[3] The distribution of copper will depend critically on the precise values of the $K_d$'s, about which there is uncertainty at the present time. Nevertheless, these calculations strongly suggest that the slow phase of enzyme reduction originates in the occupation of inhibitory sites. It is not clear whether inhibitory copper alone or catalytic copper perturbed by inhibitory copper is the source of slow reduction, but the 19% average slow-phase amplitude (Table I, Figure 3) appears more consistent with the latter possibility.

Overall, the data reported herein implicate the reduction of two catalytic coppers in a single-exponential process with a limiting rate constant of 250 $s^{-1}$. At 35 °C, pH 6.0, and 10 mM fumarate ion, DβM has a turnover number of approximately 16 $s^{-1}$ with tyramine as substrate, while turnover numbers as high as 50 $s^{-1}$ have been observed with *p*-fluorophenethylamine as a substrate (Miller & Klinman, 1985).

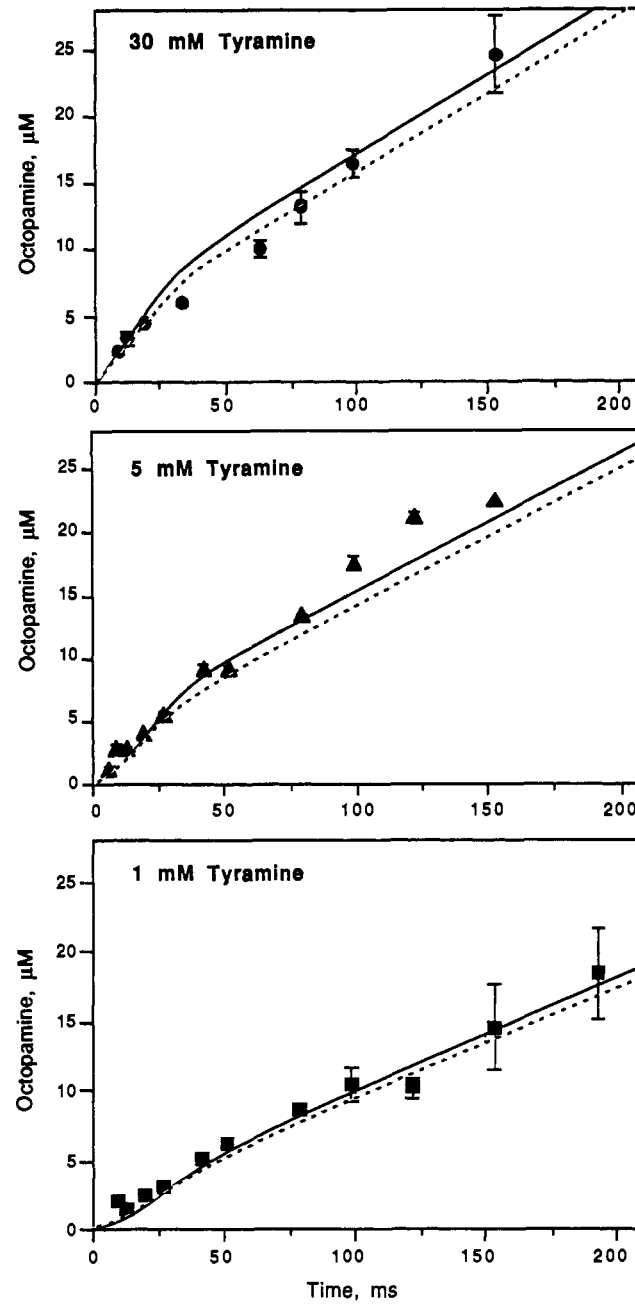



FIGURE 4: Pre-steady-state hydroxylation of tyramine. Formation of octopamine by 10 μM DβM mixed with 1, 5, and 30 mM tyramine hydrochloride is shown. Reaction conditions after mixing enzyme and substrate were 10 mM ascorbate, 100 mM chloride by the addition of KCl, 10 mM disodium fumarate, 50 mM $KP_i$, pH 6.0, and $T$ = 35 °C. Theoretical curves are from simulations based on Scheme I (dotted box), where the binary $K_d$ for tyramine is 6 times larger than the ternary $K_d$ due solely to a difference in $k_{off}$. The solid curves assume fully active enzyme and the rate constants listed in the text. The dashed curves assume 80% active enzyme due to distribution of copper into inhibitory sites and rate constants which are 20% larger than those for the solid curves.

This comparison indicates that at saturating levels of ascorbate the reduction of both catalytic copper ions is at least 5-fold faster than the overall rate of turnover of any known substrate for DβM, confirming the assumption that enzyme reduction by ascorbate is not rate determining in steady-state kinetics measurements (Ahn & Klinman, 1983; Miller & Klinman, 1985).

*Pre-Steady-State Enzyme Turnover and Kinetic Modeling.* The pre-steady-state turnover of 1, 5, and 30 mM tyramine by DβM under conditions of pH 6.0, 10 mM fumarate, and

[2] N. J. Blackburn, personal communication. The apparent difference in affinities of the two catalytic sites for $Cu^{2+}$ contrasts with copper activation studies, which imply equal affinities for $Cu^{1+}$ (Klinman et al., 1984). This descrepancy suggests different reduction potentials for the two sites (0.059 V per p$K$ unit difference). As of this writing there are no reported measurements of the reduction potential(s) of enzyme containing two $Cu^{2+}$ ions per subunit, although values of 0.31 and 0.37 V have been reported for enzyme containing only one copper (Walker et al., 1977; Ljones et al., 1978).

[3] A stoichiometry of 8.0 $Cu^{2+}$/tetramer gives a distribution with E–$Cu_a$ = 18%, E–$Cu_aCu_b$ = 61%, E–$Cu_aCu_bCu_c$ = 16%, and E–$Cu_aCu_c$ = 5%, where $Cu_a$ and $Cu_b$ are the first and second catalytic copper ions and $Cu_c$ is the inhibitory copper.

atmospheric $O_2$ is shown in Figure 4. The reaction mixtures also contained high chloride to allow comparison with steady-state parameters (Miller & Klinman, 1985). Modeling the data involved consideration of substrate binding order and the magnitude of individual rate constants in the kinetic mechanism. The use of isotope effects by Klinman and coworkers to investigate the kinetic and chemical mechanism of D$\beta$M has allowed calculation of all the rate constants in the minimal kinetic mechanism of Scheme I, with the exception of those for reduction of enzyme by ascorbate and binding of substrates to free enzyme [cf. Klinman and Ahn (1983) for application to the substrate dopamine]. Calculation of the rate constants with tyramine as a substrate was done by substituting the limiting kinetic parameters $V_{max}$, $V_{max}/K_m$ for oxygen and tyramine, the deuterium isotope effects on these parameters, and the intrinsic isotope effect on the C–H bond cleavage step, $^Dk = k_{5H}/k_{5D}$, into the equations derived by Ahn and Klinman (1983). The primary and secondary intrinsic isotope effects on C–H cleavage have been determined for dopamine to be 9.4 ± 1.3 (Miller & Klinman, 1983) and 1.19 ± 0.06 (Miller & Klinman, 1985), respectively, giving a final value of $^Dk = 11.2 \pm 1.6$; it is assumed in the calculation of rate constants for tyramine that the intrinsic isotope effect is the same as that for dopamine.[4] Average values of two determinations of the limiting kinetic parameters with tyramine as substrate [taken from Miller and Klinman (1985)] are as follows: $(V/K)_{tyramine} = (5.5 \pm 0.4) \times 10^4$ M$^{-1}$ s$^{-1}$, $(V/K)_{oxygen} = (3.31 \pm 0.03) \times 10^5$ M$^{-1}$ s$^{-1}$, $^D(V/K)_{tyramine} = 1.3 \pm 0.3$, $^D(V/K)_{oxygen} = 1.69 \pm 0.04$, $V = 15.6 \pm 2.3$, and $^DV = 1.31 \pm 0.13$, with uncertainties reflecting the range of the two measurements. Insertion of these values into the equations of Ahn and Klinman (1983) gives the following values for the rate constants in Scheme I: $k_{3,Tyr} = 5.7 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_{4,Tyr} = 16$ s$^{-1}$, $k_{3,O_2} = 3.6 \times 10^5$ M$^{-1}$ s$^{-1}$, $k_{4,O_2} = 37$ s$^{-1}$, $k_5 = 510$ s$^{-1}$, and $k_7 = 16$ s$^{-1}$.

The rate constants for tyramine and $O_2$ binding to free enzyme cannot be calculated from the limiting parameters listed above since these were obtained by extrapolation to infinite concentration of the alternate substrate. However, steady-state kinetics measurements by Kruse et al. (1986) show that the apparent dissociation constants of tyramine and $O_2$ from their binary complexes with enzyme are approximately 10-fold larger than the corresponding dissociation constants from the ternary complex (pH 6.6, in the absence of fumarate ion). As shown in the following paper (Brenner & Klinman, 1989) data are best fit assuming a 6-fold difference in $K_d$'s due solely to a difference in $k_{off}$, leading to values of $k_{1,Tyr} = 5.7 \times 10^4$ M$^{-1}$ s$^{-1}$ and $k_{2,Tyr} = 96$ s$^{-1}$.

Finally, consideration was given to the order of substrate binding. Given the small value of $^D(V/K)_{tyramine}$, which is within experimental error of unity, vs the statistically larger value for $^DV/K)_{oxygen}$, we conclude that the reaction is predominantly ordered with $O_2$ binding preceded by tyramine. A similar conclusion has been reached with regard to dopamine kinetics at pH 6.0 and 10 mM fumarate (Klinman et al., 1980) and for tyramine hydroxylation with ferrocyanide as reductant (Fitzpatrick et al., 1986). Therefore, the pre-steady-state kinetics of D$\beta$M were modeled with the ordered mechanism consisting of the upper pathway in Scheme I (dotted box). The differential equations corresponding to this mechanism are listed in eq 3–8. The reduction of both enzyme-bound copper ions by 10 mM ascorbate is represented as an irreversible step, $k = 232$ s$^{-1}$ (calculated from the data in Figure 3).

$$d[E(II)]/dt = -k_r[E(II)] + k_7(E(II)P] \quad (3)$$

$$d[E(I)]/dt = k_r[E(II)] - k_{1,Tyr}[Tyr][E(I)] + k_{2,Tyr}[E(I)Tyr] \quad (4)$$

$$d[E(I)Tyr]/dt = k_{1,Tyr}[Tyr][E(I)] + k_{4,O_2}[E(I)Tyr,O_2] - [E(I)Tyr](k_{2,Tyr} + k_{3,O_2}[O_2]) \quad (5)$$

$$d[E(I)Tyr,O_2]/dt = k_{3,O_2}[O_2][E(I)Tyr] - [E(I)Tyr,O_2](k_{4,O_2} + k_5) \quad (6)$$

$$d[E(II)P]/dt = k_5[E(I)Tyr,O_2] - k_7[E(II)P] \quad (7)$$

$$d[P]/dt = k_7[E(II)P] \quad (8)$$

The solid lines in Figure 4 are simulations of the time dependence of the formation of the sum of E(II)P and P based on integration of eq 3–8 (see Materials and Methods for details of the numerical integration). Curvature of these lines is characterized by a short initial lag (corresponding to sequential binding of the two substrates) followed by a rapid increase in product formation (due to the rapid C–H bond cleavage step) which levels out at the relatively slow steady-state rate (predominantly determined by the rate of product release). As discussed under Reduction of Enzyme-Bound Copper, binding of copper to a third, inhibitory site is expected to reduce the concentration of enzyme containing two catalytic coppers to only 80% of $E_T$, whereas the calculated rate constants discussed above were based on the assumption of 100% active enzyme. We therefore resimulated the transients in Figure 4 assuming 80% active enzyme and using calculated rate constants 20% larger than those used initially, although the value of $k_r$ was held fixed at 232 s$^{-1}$ since this rate constant was measured directly by the rapid freeze-quench method. As shown by the dashed lines in Figure 4, this small correction has only a modest effect on modeled transients. The sensitivity of the simulated curves to changes in $k_5$ is also not very great due to the large rate constant for C–H bond cleavage relative to oxygen binding. Additionally, expanding the ordered kinetic mechanism into a random one increases the rate of product formation only slightly for tyramine concentrations of ≥1 mM (not shown). The key parameters in determining the goodness of fit are the rate constants for oxygen binding and product release. On balance, the agreement between experimental data and simulations is quite good and provides support for the ordered pathway in Scheme I. We note that this is the second example of a comparison of measured pre-steady-state rate parameters with values calculated from steady-state isotope effects. In a previous study, Palcic and Klinman (1983) contrasted stopped-flow data for the plasma amine oxidase catalyzed oxidation of aromatic amines with calculated rate constants using deuterium isotope effects on $V_{max}$ and $V_{max}/K_m$. Analogous to the present study, excellent agreement between measured and calculated constants was obtained.

*Oxidation State of Enzyme-Bound Copper in the Approach to the Steady State.* When oxidized enzyme is mixed simultaneously with ascorbate and tyramine, the copper EPR signal should reflect the sum of the concentrations of all the enzyme forms containing $Cu^{2+}$, i.e., free oxidized enzyme and the oxidized enzyme–product complex, each containing two ions of $Cu^{2+}$ per active site. When the steady state is reached, the enzyme–product complex will be the predominant enzyme form since the rate of product release (16 s$^{-1}$) is much slower than the preceding steps in the mechanism (enzyme reduction, substrate binding, and C–H bond cleavage). Therefore, the steady state should be characterized by a relatively large $Cu^{2+}$ EPR signal. A more quantitative prediction of the steady-state EPR signal is obtained by simulating the time dependence of

[4] As discussed in some detail (Miller & Klinman, 1985), this assumption is a good one for a range of phenethylamine substrates and is expected to have little impact on calculated rate constants.

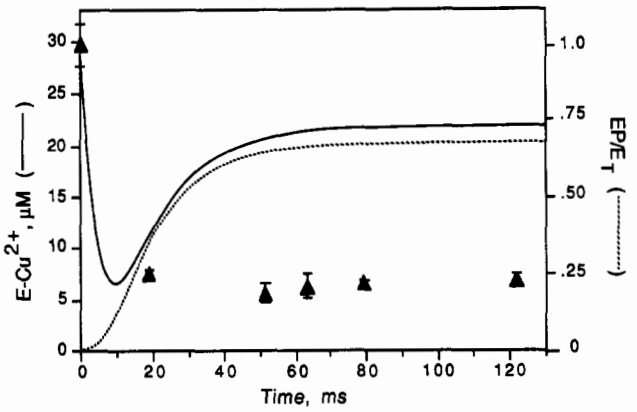



FIGURE 5: Disappearance of the DβM copper EPR signal in the steady state. The theoretical time dependence of the enzyme's $Cu^{2+}$ EPR signal (solid curve) and the fraction of enzyme existing as the enzyme–product complex (dashed curve) are shown with experimental data (triangles). Reaction conditions were as follows: 15 μM DβM (2 $Cu^{2+}$/subunit), 5 mM tyramine hydrochloride, 10 mM ascorbate, 10 mM disodium fumarate, 95 mM KCl, 50 mM $KP_i$, pH 6.0, and $T$ = 35 °C. Theoretical curves were obtained by simulation as described under Figure 4 and in the text, assuming fully active enzyme.

the oxidized enzyme forms as was done for the product formation transients discussed above. Results of these simulations for 5 mM tyramine are shown in Figure 5 for the expected total magnitude of the $Cu^{2+}$ EPR signal (solid line) and for the expected signal arising from the enzyme–product complex alone (dashed line). A rapid drop in the EPR signal to ~25% of the initial value due to enzyme reduction by ascorbate (232 $s^{-1}$) was expected to be followed by a return of the signal to ~70% of the total copper, corresponding to formation of the steady-state level of oxidized enzyme–product complex. Consideration of copper distribution into inhibitory sites (see above) and the presence of some nonreducilbe copper do not significantly alter these predictions. Contrary to our expectations, however, when oxidized enzyme was mixed simultaneously with ascorbate and tyramine, the intensity of the copper EPR signal dropped to about 19% of the its initial value within 15 ms and remained there in the steady state (Figure 5). A similar level of oxidized copper was observed in the presence of 10 mM ascorbate alone (100 mM $Cl^-$). The large discrepancy between the predicted and observed levels of oxidized copper during steady-state catalysis was initially very surprising. Our success in modeling the product formation transients supports the expectation that the enzyme–product complex is the predominant steady-state enzyme form, but the large copper EPR signal expected for this species is virtually absent from the freeze-quenched samples. There are two plausible explanations for this result. Either the enzyme–product complex involves a binuclear copper center in which antiferromagnetic coupling eliminates the characteristic EPR signal of $Cu^{2+}$ of free enzyme [as in proteins such as hemocyanin (Solomon, 1981)], or the oxidized copper in the enzyme–product complex becomes reduced by ascorbate to diamagnetic, EPR-silent $Cu^{1+}$. In the accompanying paper (Brenner & Klinman, 1989), single-turnover studies show that the enzyme–product complex contains $Cu^{2+}$, which is fully detectable by EPR in the absence of excess ascorbate. This result argues against binuclear copper in the enzyme–product complex, implicating ascorbate reduction of $Cu^{2+}$ to $Cu^{1+}$ *prior* to product dissociation.

## Conclusions

Three major conclusions have been reached in this study concerning the mechanism of dopamine β-monooxygenase. First, we have shown that two catalytic coppers per subunit are reduced by ascorbate in a single-exponential process with a limiting rate constant (250 $s^{-1}$) that is fully compatible with turnover kinetics. Turnover of enzyme in the presence of excess ascorbate has been monitored with rapid acid- and freeze-quench techniques, allowing observation of transients for formation of hydroxylated product and bound $Cu^{2+}$, respectively. Product formation has been found to behave as predicted and can be modeled by the mechanism in Scheme I, with measured rate constants for enzyme reduction by ascorbate and calculated rate constants for the interaction of substrate and oxygen with reduced enzyme. By contrast, the $Cu^{2+}$ EPR signal for enzyme–product complex failed to reappear as expected. This phenomenon, which is examined in greater detail in the following paper, provides the first direct evidence in support of distinct binding sites for reductant vs substrate to dopamine β-monooxygenase.

## Acknowledgments

We are indebted to Dr. Alan Bearden of the University of California at Berkeley for assistance with EPR instrumentation and to Dr. Dale Edmondson for his initial assistance with rapid freeze-quench methodology.

**Registry No.** DβM, 9013-38-1; Cu, 7440-50-8; ascorbic acid, 50-81-7; tyramine, 51-67-2.

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# Correlation of Copper Valency with Product Formation in Single Turnovers of Dopamine β-Monooxygenase†

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ABSTRACT: Chemical- and freeze-quench EPR techniques have allowed single-turnover studies of the copper-containing enzyme dopamine β-monooxygenase. Reduction of enzyme by a stoichiometric amount of ascorbate followed by rapid mixing with tyramine leads to oxidation of bound copper and formation of hydroxylated product in the expected 2:1 ratio. The tyramine dependence of single turnovers yields a limiting rate of 82 ± 9 $s^{-1}$ and $K_m$ of 3 ± 1 mM, in agreement with kinetic modeling based on steady-state parameters. Together these results show that the reduced enzyme is a catalytically competent species, with bound copper acting as the sole reservoir of reducing equivalents. The correlation of copper oxidation and substrate hydroxylation rules out significant antiferromagnetic spin coupling in the enzyme–product complex. Since the enzyme–product complex's $Cu^{2+}$ EPR signal is absent in the transient approach to the steady state [Brenner, M. C., Murray, C. J., & Klinman, J. P. (1989) *Biochemistry* (preceding paper in this issue)], this result implies that ascorbate reduces copper in the enzyme–product complex. These findings have important consequences for catalysis and active site structure in dopamine β-monooxygenase.

Dopamine β-monooxygenase (DβM, EC 1.14.17.1) is a copper-containing enzyme catalyzing the hydroxylation of dopamine to norepinephrine concomitant with a two-electron oxidation of ascorbic acid. The number of copper ions constituting an active site and the role played by enzyme-bound copper in the redox events of catalysis have been major questions in understanding this enzyme's function. Early work by Friedman and Kaufman (1965) established the ability of enzyme-bound copper to undergo changes in valence due to the presence of either ascorbate or substrate. These results suggested a ping-pong mechanism for enzyme turnover involving multiple copper centers as the repository for the reducing equivalents required for catalysis. In subsequent studies, however, evidence was put forth for the presence of only a single tightly bound copper ion per subunit (Skotland et al., 1980; Skotland & Flatmark, 1983; Skotland & Ljones, 1979; Syvertsen et al., 1986). Importantly, prereduced enzyme was shown to be incompetent to hydroxylate substrate in the absence of additional reductant, leading to the suggestion of ternary complex formation between reduced enzyme, substrate, and ascorbate (Skotland et al., 1978).

Our perspective on dopamine β-monooxygenase changed markedly in 1984 with the demonstration that two copper ions

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