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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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per subunit are required in DβM turnover (Klinman et al., 1984) and in mechanism-based inactivation (Ash et al., 1984). Subsequent steady-state measurements on the role of reductant have clearly demonstrated ping-pong kinetics for enzyme reduction and substrate binding (Fitzpatrick et al., 1986; Stewart & Klinman, 1987). Additionally, rapid freeze-quench studies with enzyme containing two Cu^{2+} ions per subunit now show that both Cu^{2+} ions are reduced simultaneously by ascorbate at a rate consistent with enzymatic catalysis, supporting a ping-pong mechanism and the participation of both coppers in the redox process (Brenner et al., 1989).

A surprising feature of the freeze-quench studies described in our recent report (Brenner et al., 1989) is the very low EPR[1] signal for enzymic copper in the transient approach to the steady state, contrasting with the results of computer modeling which indicate that the oxidized enzyme–product complex is the predominant enzyme form. This finding introduces the possibility of EPR-silent antiferromagnetically spin coupled coppers in the E–P complex, in contrast to previous EPR studies of resting forms of DβM which revealed no evidence for magnetic interactions (Villafranca, 1981; Brenner, 1988; McCracken et al., 1988).

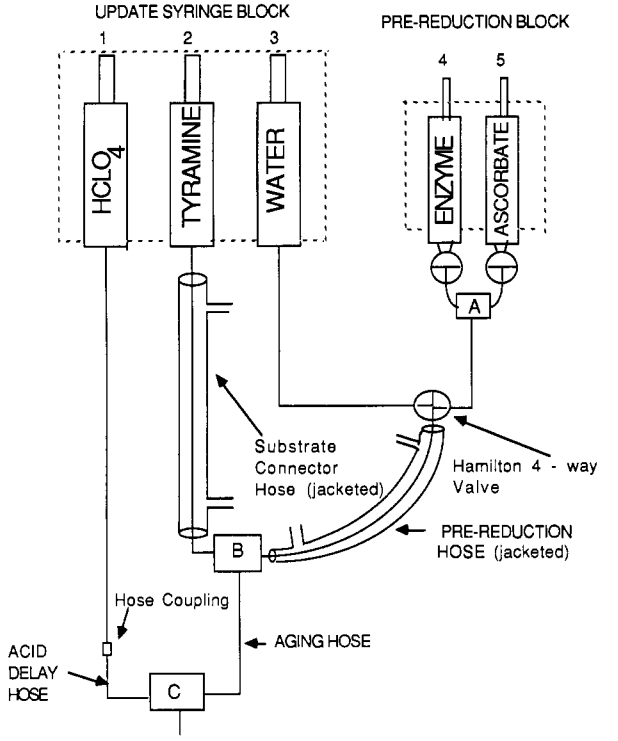
In order to resolve the origin of the steady-state EPR characteristics of DβM, a series of single-turnover studies were initiated in which prereduced DβM was mixed with substrate in the absence of excess reductant. These experiments permit a direct correlation of product formation with the redox state of enzyme-bound copper showing, first, that prereduced enzyme is a viable catalytic complex and, second, that the enzyme–product complex contains a fully detectable EPR Cu^{2+} signal. The latter result rules out antiferromagnetic spin coupling between Cu^{2+} ions in E–P, leading us to conclude that ascorbate can reduce oxidized copper in E–P prior to product dissociation. This conclusion contrasts with all previous models for DβM which invoke an interaction of reductant only with the free enzyme form [e.g., Villafranca (1981) and Ljones and Skotland (1984)]. The consequences of these findings to catalysis and active site structure in dopamine β-monooxygenase are discussed.

Materials and Methods

All reagents, method of DβM isolation, determination of copper, and computer simulation of product formation transients were as described in the previous paper. EPR measurements were the same as in the previous paper (Brenner et al., 1989) with the following settings; 5-mW microwave power, 20-G modulation width, 2970 ± 500 G field sweep, 57 s/scan × four scans, 0.3-s time constant, and $T \cong 15$ K.

Rapid Mixing with Prereduced Enzyme. Since stoichiometrically reduced DβM partially reoxidizes in air in a matter of minutes, it was necessary to construct an apparatus that allowed rapid mixing of enzyme and substrate immediately following stoichiometric reduction of enzyme with ascorbic acid. To this end, a water-thermostated syringe block and a hand-pushed ram, fitted with two 2.5-mL Hamilton gas-tight syringes, was constructed so that a preset volume of enzyme and ascorbate could be passed through an Update Instruments mixer and a four-way valve (No. 86729, Rainin Instrument Co.) into a water-jacketed Teflon storage hose. A schematic of the arrangement of syringes, hoses, mixers, and valves required to carry out these experiments is shown in Scheme I. In a typical experiment the jacketed prereduction hose (detached from mixer B) was filled with equal volumes of 60 μM

Scheme I: Apparatus for Prereduction and Rapid Quenching of Dopamine β-Monooxygenase



DβM subunits and 60 μM ascorbic acid from the syringes in the prereduction block. The position of the four-way valve was then switched to connect syringe 3 to the prereduction hose, this hose was connected to mixer B, and the reduced enzyme (30 μM) was allowed to sit at 35 °C until 43 s had elapsed after mixing. The reduced enzyme in the prereduction hose was then rapidly expelled by water from the Update Instruments ram-driven syringe (no. 3) and mixed one-to-one with substrate or buffer into an aging hose whose volume determined the reaction time. The enzyme–substrate mixture was rapidly quenched by mixing with 0.75 M $HClO_4$ from syringe 1 or by rapid freezing in −135 °C isopentane (see the previous paper for details of HPLC product analysis and rapid freeze-quenching procedures). In experiments involving oxygen concentrations different from air saturation, the Teflon substrate connector hose was replaced by stainless steel tubing (Rainin Instrument Co.). The tyramine stock solution was bubbled with either O_2(g) or N_2(g) for two h at 35 °C in a polypropylene test tube fitted with a serum cap and inlet and outlet needles. The base of a 12-in. stainless needle was connected to the substrate connector hose and the tip inserted into the test tube containing the tyramine stock solution. The Update syringe (no. 2) was purged with O_2(g) or N_2(g) by eight cycles of filling the syringe and expelling its contents into the air, prior to filling with the N_2- or O_2-saturated tyramine stock. The needle was then removed, and the substrate connector hose was attached to mixer B. Octopamine formation single-turnover rates were determined from nonlinear least-squares fits to eq 1 with the program SYSTAT from SYSTAT Inc., Evanston IL.

$$[Oct]_t = [Oct]_\infty(1 - e^{-kt}) \quad (1)$$

Results

Turnover of Tyramine by Prereduced Enzyme. Contrary to the results of Skotland et al. (1978), who saw little turnover by prereduced DβM containing one copper ion per subunit, enzyme with two copper ions per subunit can be reduced to

[1] Abbreviations: EPR, electron paramagnetic resonance; HPLC, high-pressure liquid chromatography.

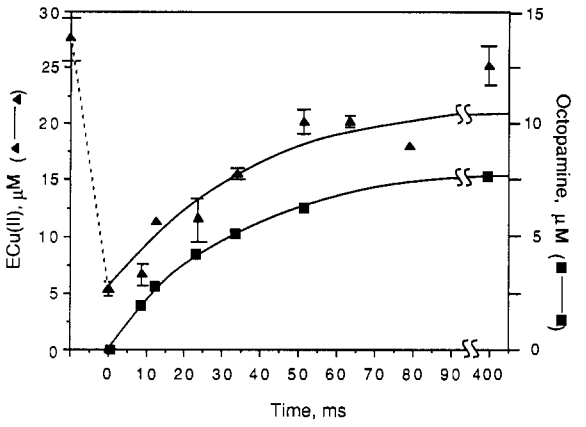


FIGURE 1: Correlation of octopamine formation and copper oxidation during a single turnover by dopamine β-monooxygenase. (■) Concentration of octopamine in acid-quenched samples of 15 μM reduced DβM which had reacted for the indicated times with 5 mM tyramine hydrochloride. (▲) Concentration of E–Cu^{2+} in freeze-quenched samples. The initial value at 27.5 μM E–Cu^{2+} refers to the amplitude of the $g_\perp$ EPR signal from samples in which the ascorbate and tyramine solutions were replaced by buffer blanks. The value of E–Cu^{2+} at zero milliseconds refers to the signal from prereduced enzyme rapidly mixed with buffer instead of tyramine. Error bars indicate the range for the average of duplicate samples. Experimental conditions were as described under Materials and Methods. Additionally, the conditions after mixing enzyme and tyramine were 15 μM DβM subunits, 10 mM disodium fumarate, 100 mM KCl, 50 mM KP_i, pH 6.0, and T = 35 °C.

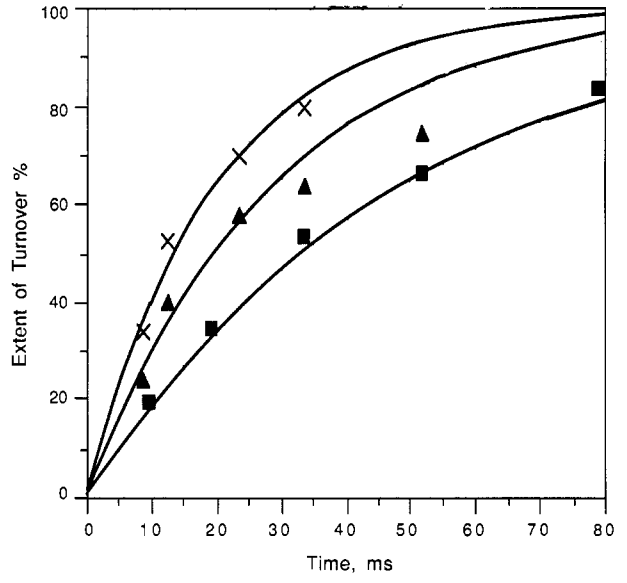


FIGURE 2: Single turnovers of DβM: 1–30 mM tyramine. Octopamine formation by 15 μM DβM at 1 (■), 2 (▲), and 30 mM (×) tyramine are shown. The data were normalized so that the amount of octopamine formed at 400 ms corresponds to 100% on the y axis for each tyramine concentration. Solid curves are single-exponential fits with rates of 21, 33, and 59 s^{-1} based on nonlinear regression. Experimental conditions are the same as in Figure 1 except for the tyramine concentration.

yield a catalytically active species. As shown in Figure 1 stoichiometric reaction of enzyme with ascorbic acid for 43 s leads to the initial disappearance of 80% of the enzyme-bound Cu^{2+} EPR signal. The actual time required for reduction is only a few seconds, but manipulation of the valves and hose couplings necessitated a longer period of incubation. Since high levels of ascorbate typically fail to reduce the last 10–20% of the enzyme-bound copper (Brenner et al., 1989), the presence of nonreducible copper is expected. Upon reaction of the reduced enzyme with 5 mM substrate, 70% of the reducible copper undergoes conversion to EPR-detectable

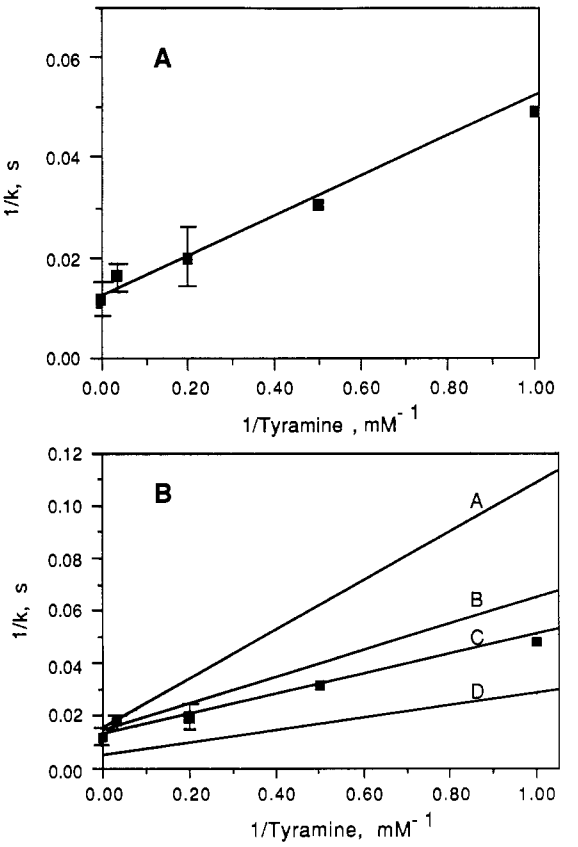
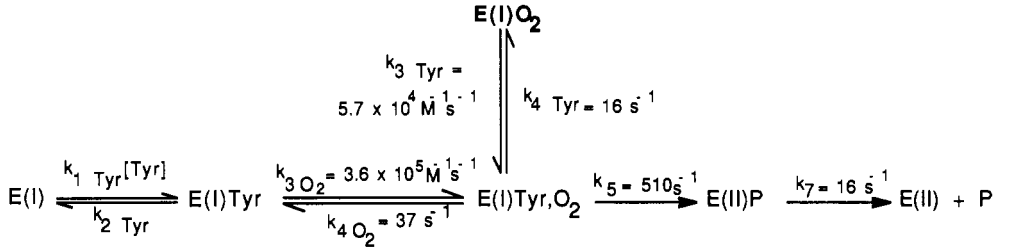


FIGURE 3: Tyramine dependence of DβM single turnovers. (A) Experimental conditions were the same as in Figure 1 except at 0.4 M tyramine hydrochloride the chloride concentration was 0.4 M instead of 0.1 M. The solid line is a nonlinear least-squares fit based on the program HYPER by Cleland (1979), yielding a maximal rate of 82 ± 9 s^{-1} and a half-maximal rate at 3 ± 1 mM tyramine. (B) Theoretical tyramine dependence of DβM single turnovers. Ratios of K_d(binary) to K_d(ternary) and the rate constant (k_{on} or k_{off}) responsible for the difference in K_d's are as follows: line A, 6:1 (k_{on}); line B, 10:1 (k_{off}); line C, 6:1 (k_{off}); line D, 1:1.

Cu^{2+}. Octopamine product is formed in parallel with copper reoxidation, giving a net formation of 7.5 μM octopamine and 15.5 μM Cu^{2+}. A nonlinear regression fit of the data to a single exponential yields first-order rate constants of 31 ± 6 s^{-1} and 35 ± 1 s^{-1} for enzyme reoxidation and product formation, respectively. The product formation studies were repeated at 1, 2, 30, and 400 mM tyramine; representative data are shown in Figure 2, and the accumulated rates and extents of enzyme turnover are presented in Table I. As shown, the turnover rate increases nearly 5-fold on going from 1 to 400 mM tyramine, with an average fractional turnover of 0.52, the lowest and highest fractional turnovers observed being 0.38 and 0.72. A reciprocal plot of the turnover rates and tyramine concentrations yields a limiting rate of 82 ± 9 s^{-1} at saturating tyramine and a half-maximal rate at 3 ± 1 mM tyramine (Figure 3A) in comparison to a steady-state K_m of 0.3 mM (Miller & Klinman, 1985). The ability of prereduced enzyme to hydroxylate tyramine simultaneous with oxidation of enzyme-bound copper on the millisecond time scale shows that enzyme with two reduced copper ions contains all the reducing equivalents required for the hydroxylation reaction. The 82-s^{-1} limiting rate of turnover and the apparent K_m of 3 mM were initially surprising (Brenner & Klinman, 1987) but, as discussed below, are entirely consistent with steady-state measurements.

Effect of O_2 on DβM Single Turnovers. To examine the effect of oxygen concentration on DβM single turnovers, prereduced enzyme was mixed with tyramine stock solutions

Scheme II: Kinetic Mechanism for Tyramine Hydroxylation by Reduced DβM[a]



[a] As described in the text, preincubation of reduced enzyme with air is expected to lead to some formation of $E(I)O_2$ prior to mixing with tyramine; the level of $E(I)O_2$ and the magnitudes of $k_{1,Tyr}$ and $k_{2,Tyr}$ depend on the relationship between binary and ternary dissociation constants for the interaction of O_2 and tyramine with enzyme.

Table I: Tyramine Dependence of Single Turnovers of Prereduced DβM[a]

tyramine (mM)	k (s^{-1})	Oct_∞/E_t[b]	% turnover[c]	k_{av} (s^{-1})
1.0	21 ± 1	0.60	75	
2.0	33 ± 2	0.39	49	
5.0	43 ± 4	0.38	48	
5.0	73 ± 8	0.56	70	49 ± 14
5.0	35 ± 1	0.51	64	
30	58 ± 4	0.46	57	59 ± 1
30	59 ± 1	0.44	55	
400	101 ± 11	0.60[d]	75	
400	101 ± 6	0.65	81	92 ± 16
400	101 ± 10	0.41	51	
400	65 ± 1	0.72	90	
average:		0.52 ± 0.11	65 ± 14	

[a] Results are from three different enzyme preparations, with the scatter in fractional turnovers occurring within as well as between preparations. [b] Extent of product formation at 400 ms. [c] Based on 80% reducible copper and observed Oct_∞/E_t ratios. [d] Extent of product formation at 250 ms.

in which O_2 had been either depleted or increased, resulting in final O_2 concentrations in reaction mixtures of approximately 0.10 or 0.6 mM. Reaction of prereduced enzyme with O_2-saturated tyramine solutions led to an unexpected 2-fold decrease in the extent of turnover. This effect was not investigated further although enzyme inhibition at high oxygen tension in the steady state has also been observed in this laboratory (unpublished results). Whatever the source of the decreased amount of turnover, insufficient data were collected to establish a change in rate at the elevated oxygen tension. By contrast, decreasing the O_2 concentration to 0.10 mM produced a clear diminution in rate from 59 ± 1 to 34 ± 3 s^{-1} at 30 mM tyramine.

Computer Modeling of Single DβM Turnovers. DβM single turnovers were modeled according to Scheme II. This is similar to the ordered binding pathway of Scheme I in Brenner et al. (1989) except that the rate constant for enzyme reduction is no longer required; additionally, since air was not excluded during enzyme incubation with ascorbate, allowance has been made for some binding of O_2 to prereduced enzyme prior to mixing with tyramine. As discussed in Brenner et al. (1989), precise values of $k_{1,Tyr}$ and $k_{2,Tyr}$ [as well as the level of $E(I)O_2$] are not available, although these can be estimated from measured ternary dissociation constants (Miller & Klinman, 1985) and the relationship of binary to ternary dissociation constants previously reported (Kruse et al., 1986). However, differences in experimental conditions between Kruse et al. (1986) and the present study introduced uncertainty in the ratio of K_d(binary) to K_d(ternary). We therefore simulated product formation transients with ratios of these dissociation constants varying from 1:1 to 10:1 using differential equations corresponding to Scheme II and the method of numerical integration discussed in Brenner et al. (1989). From the time course for formation of the sum of E(II)P and P at tyramine concentrations of 1, 2, 5, and 30 mM, first-order rate constants were estimated. Reciprocal plots of these theoretical rate constants are shown in Figure 3B for comparison with experimental data (Figure 3A). Identical binary and ternary substrate dissociation constants give a poor fit to the experimental data (line D, Figure 3B) with a predicted limiting rate at saturating tyramine of 200 s^{-1}, 3 times larger than the experimental value. A 10-fold difference in binary and ternary dissociation constants gives a limiting turnover rate of 70 s^{-1}, in agreement with the experimental data (line B, Figure 3B). However, a better fit at the low-substrate end of the reciprocal plot is obtained by assuming K_d(binary) = $6K_d$(ternary), with the entire difference in dissociation constants arising from k_{off} (line C, Figure 3B). By contrast, assignment of this 6-fold difference in dissociation constants to k_{on} for tyramine binding to free enzyme gives a poor fit to the data at low (1 mM) tyramine concentration (line A, Figure 3B). This disparity is greater for a 10-fold difference in K_d's due solely to a difference in k_{on} (not shown). As discussed by Brenner et al. (1989), distribution of copper into a third inhibitory site is expected to diminish the active enzyme concentration to 80% of total enyzme, resulting in a 20% increase in calculated rate constants. Remodeling of the data in Figure 3 following consideration of this point led to theoretical lines which were only modestly different from those shown.

Discussion

Reduced DβM Is Catalytically Competent. Early experiments implied the catalytic competence of reduced DβM (Friedman & Kaufman, 1965) but were criticized for a number of reasons, which included a lack of kinetic evidence required for a true demonstration of catalytic competence, the possible presence of enzyme-bound ascorbic acid contaminating the reduced enzyme (Skotland et al., 1978), and the very small amount of turnover (~20%) carried out by reduced enzyme. Skotland et al. (1978) undertook similar experiments, observing only 7% turnover after 1 min of incubation of reduced enzyme with substrate. However, their enzyme preparation contained 2.52 coppers per tetramer, 32% of the total required for full catalytic activity. This copper to enzyme stoichiometry will yield only 10% of the subunits in an active state because of the random distribution of copper among the eight catalytic copper binding sites per tetramer (Klinman et al., 1984). At the optimal copper to subunit stoichiometry of eight coppers per tetramer employed herein, the fraction of enzyme which turns over (Table I) is 7-fold greater than observed by Skotland and co-workers.

As shown in the present study, DβM with the full complement of catalytic copper can be reduced by ascorbate, followed by copper reoxidation and substrate hydroxylation at the expected ratio of 2:1 (Figure 1). However, even at the optimal copper stoichiometry, fractional turnovers fall short

of 100%. Incubation of 15 μM subunits with 1 equiv of ascorbate leaves approximately 20% of the enzymic copper oxidized; subsequent incubation of the reduced enzyme with substrate leads to reoxidation of 70% of the reducible copper, Figure 1, and substrate hydroxylation which is 65% of total enzyme after correction for the fraction of reducible copper, Table I. As discussed (Brenner et al., 1989), a fraction of enzyme is reduced by excess ascorbate in a slow phase, attributed to the distribution of copper among catalytic and inhibitory sites. During reoxidation of stoichiometrically reduced enzyme, similar behavior is anticipated. In this instance the relevant binding constants for copper refer to Cu^{1+} and can be estimated from previous turnover studies indicating equal dissociation constants for two catalytic copper binding sites ($pK \geq 8.0$) and $pK = 5.3$–5.9 for an inhibitory site (Klinman et al., 1984). Under these conditions 80% of total enzyme is expected to contain two coppers at the catalytic sites, with the remaining 20% of the enzyme containing copper at an inhibitory site or lacking copper in a catalytic site. The observed reoxidation of 70% of the reducible copper, Figure 1, and formation of a comparable amount of hydroxylated product, 65 ± 14%, Table I, agree nicely with these predictions.

In addition to the above considerations, demonstration of true catalytic competence requires that reduced enzyme behave kinetically as anticipated from steady-state parameters. In principle, the maximal rate of a single turnover of DβM is the rate of C–H bond cleavage (510 s^{-1}) when tyramine is saturating and the reduced enzyme has O_2 fully bound. For this reason, the observed limiting value of 82 s^{-1} was initially interpreted in terms of a conformational change following enzyme reduction to a form with diminished catalytic efficiency (Brenner & Klinman, 1987). However, subsequent computer modeling and comparison with pre-steady-state data at high ascorbate (Brenner et al., 1989) and stoichiometric ascorbate (this study) have shown that turnover can be fit by allowing for relatively weak O_2 and tyramine binding in the binary enzyme–substrate complexes. When the binary and ternary substrate dissociation constants are assumed to be identical, over 60% of the prereduced enzyme is calculated to have O_2 bound prior to mixing with tyramine, leading to a predicted limiting turnover rate of 200 s^{-1}, nearly 3-fold faster than the observed value (line D, Figure 3B). In contrast, when a 6-fold difference between binary and ternary dissociation constants is assumed, the fraction of reduced enzyme with O_2 bound is only 24%. At saturating tyramine concentration, turnover by the remaining 76% of enzyme is rate limited by O_2 binding (73 s^{-1}) and C–H bond cleavage (513 s^{-1}). Thus, for the bulk of the reduced enzyme, *the relevant steady-state rate constant for comparison with pre-steady-state single turnovers is* $(V/K)_{oxygen}$ *for reaction of* O_2 *with E–tyramine; in air-saturated solution (0.21 mM* O_2*) this value is 70* s^{-1}*, in close agreement with the observed limiting rate of 82 ± 9* s^{-1} *(line C, Figure 3B)*. Furthermore, in O_2-depleted solution (0.10 mM O_2), the theoretical rate of turnover at 30 mM tyramine is expected to drop from 70 to 40 s^{-1}, in comparison with the measured decrease from 59 to 34 s^{-1}, confirming the almost complete rate limitation of DβM single turnovers by O_2 binding.

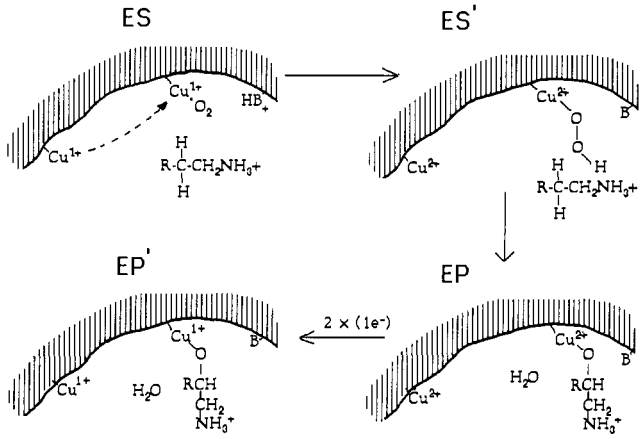
As the above discussion indicates, the reduced form of DβM containing a full complement of copper is a kinetically competent species which couples the hydroxylation of substrate with the oxidation of the two bound Cu^{1+} ions. For this reason we find the recently reported presence of pyrroloquinoline quinone in DβM unexpected (van der Meer et al., 1988). From the data reported herein, there is no evidence that a redox cofactor other than bound copper is required in substrate hydroxylation. Although we cannot exclude a role for pyrroloquinoline quinone in the facilitation of substrate binding or electron transfer, our data preclude an organic cofactor as an obligatory repository of reducing equivalents in the DβM reaction. Additionally, our finding that the reduced enzyme is catalytically competent supports the view that spectroscopic studies of the Cu^{1+} form of DβM [cf. Scott et al. (1988)] will pertain to a viable catalytic species, rather than to a conformationally altered form as previously suggested (Brenner & Klinman, 1987).

Reduction of the Enzyme–Product Complex. In the previous paper (Brenner et al., 1989) we found a surprisingly low level of EPR-detectable copper during steady-state catalysis by DβM. This result required either that the enzyme–product complex involve spin coupling between adjacent Cu^{2+} centers or that copper in this enzyme form be reduced by ascorbate to Cu^{1+}. A low level of EPR-detectable Cu^{2+} in the steady state (~20%) was also previously observed by Skotland et al. (1980); however, these workers failed to reconstitute the isolated enzyme with the two coppers per subunit required for full catalytic activity. The experiments presented in the present paper using two copper per subunit enzyme and rapid-mixing techniques to control times for enzyme incubation with tyramine and stoichiometric ascorbate have permitted measurement of rate constants for formation of both octopamine and E–Cu^{2+}. As shown, reoxidation of E–Cu^{1+} and hydroxylation of tyramine parallel one another, and the substrate dependence of single-turnover rates is consistent with the rate constants obtained from steady-state measurements. If there were significant coupling between copper centers in the E–P complex, a large discrepancy between product formation and apparent copper reoxidation rates would be evident.

Given the absence of antiferromagnetic coupling of copper centers in the enzyme–product complex, the lack of a copper EPR signal during steady-state catalysis with high concentrations of ascorbate (10 mM) (Brenner et al., 1989) implies that ascorbate reduces Cu^{2+} in this enzyme form to Cu^{1+}. Although previous steady-state studies have concluded that ascorbate interacts only with the free enzyme, the observation of ping-pong kinetics is fully compatible with reduction of the enzyme–product complex since the chemical hydroxylation step is irreversible (Miller & Klinman, 1983). It should be noted that Fitzpatrick et al. (1986) report the same value of $(V/K)_{ascorbate}$ with several phenethylamine substrates. These observations may simply indicate an insensitivity of the rate of E–P reduction to product structure, a likely possibility given the much faster rate for reduction of free enzyme (250 s^{-1}; Brenner et al., 1989) relative to product release (16 s^{-1}; Miller & Klinman, 1985). At low ascorbate concentrations, the rate of product release may be faster than E–P reduction, making free enzyme the site of copper reduction. It is of interest to speculate about a mechanistic function for reduction of copper in the enzyme–product complex. A combination of structure–reactivity correlations and pH studies has led to a model involving inner-sphere coordination of the alkoxide product to Cu^{2+} (Ahn & Klinman, 1983; Miller & Klinman, 1985). Conceivably, reduction of Cu^{2+} to Cu^{1+} could facilitate product release by weakening the metal–alkoxide interaction. If this is true, release of product from oxidized enzyme may be extremely slow, making E–P the site of copper reduction, regardless of ascorbate concentration.

Distinct Copper Sites with Separate Functions. At the current time, no spectroscopic evidence exists for significant magnetic interaction between copper centers in DβM, either

Scheme III: Postulated DβM Mechanism Involving Distinct Copper Sites with Separate Functions[a]



[a] A reductant site and a substrate binding site with magnetically isolated copper centers are indicated. Electron transfer between coppers (dotted arrow) allows two-electron reduction of oxygen. A copper–hydroperoxide species hydroxylates the substrate (R = phenyl), leaving a Cu^{2+}–alkoxide product and H_2O. Addition of two electrons through the reductant site copper gives a Cu^{1+}–E–P complex.

in the resting form of enzyme (Villafranca, 1981; Brenner, 1988; McCracken et al., 1988) or in the enzyme–product complex (this study). These observations raise a central question regarding DβM mechanism and structure: How can two copper ions physically insulated from one another to prevent magnetic interaction constitute a site for the binding and subsequent activation of dioxygen? On the basis of an extensive analysis of reductant binding and electron transfer, Stewart and Klinman (1987) have suggested separate functions for the two copper ions. One copper is proposed to be at the core of a "reductant site" where ascorbate, ferrocyanide, or other reductants bind and deliver one electron at a time. Direct interaction of reductants with enzyme-coordinated copper has not been demonstrated, and in principle, reductants could bind in a protein cleft distinct from both metal sites. The second copper, at a distance greater than 4 Å, is the "active site" copper where oxygen binding and activation take place. This model obviates the steric crowding inherent in a complex involving simultaneous coordination of both the hydroxylated alkoxide product and reductant to a single site.

A diagram illustrating two distinct copper sites with separate functions in the catalytic mechanism is shown in Scheme III. As illustrated, the fully reduced enzyme binds oxygen and phenethylamine substrate, followed by electron transfer from both copper ions to dioxygen. The requirement for a proton in catalysis suggests protonation of the peroxide anion to give mononuclear copper–hydroperoxide (Ahn & Klinman, 1983). The subsequent mechanism of C–H cleavage and oxygen atom insertion via a concerted homolytic cleavage of the O–O and C–H bonds to give a benzylic radical which collapses to alkoxide-bound product and H_2O has been discussed by Miller and Klinman (1985). Both coppers of the enzyme–product complex are then reduced to Cu^{1+} with the reductant site copper acting as an electron shuttle. Since the inter-copper electron-transfer process is a substep of C–H bond cleavage (k_5 = 510 s^{-1} in Scheme II), electron transfer between copper centers in DβM is at least this rapid. Electron transfer between metal centers in proteins is currently an area of intense investigation (Mayo et al., 1986; McClendon, 1988), with observed rates that are faster as well as slower than those in DβM depending on donor/acceptor distance, reaction free energy, and medium effects. Perhaps more interesting than the exact rate of electron transfer in DβM is the timing of this process to ensure the proper chemistry between reactants at the active site.[2]

In known copper coordination complexes (Karlin et al., 1987) and in the proteins tyrosinase and hemocyanin (Solomon, 1981), the two-electron reduction of O_2 to a metal–peroxide involves binuclear copper where both metal centers coordinate to O_2. Mononuclear copper complexes which catalyze oxidation of organic substrates have not been adequately pursued, unlike corresponding models for cytochrome P-450 chemistry and other transition metal complexes (Sheldon & Kochi, 1981). For this reason, the proposed mechanism in Scheme III for activation of oxygen by mononuclear copper is unprecedented. In recent literature, some evidence has been advanced for the involvement of a hydroperoxide in copper-catalyzed oxygenation reactions. For example, Karlin et al. (1987) have described a phenoxo-bridged dicopper(I) compound which is capable of reversibly binding O_2 to form a peroxo–dicopper(II) complex. Following protonation to yield a postulated copper–hydroperoxide species, oxidation of triphenylphosphine to phosphine oxide occurs, reminiscent of the sulfoxidation and selenoxidation reactions carried out by DβM (May et al., 1987, 1980). However, unlike DβM, the bridged dicopper(II) complex is EPR silent due to the proximity of the coppers and bridging ligands. As discussed by Stewart and Klinman (1988), selected features of cytochrome oxidase may be relevant to the proposed mechanism of DβM. In the former protein, a binuclear heme iron–Cu center serves as the O_2 binding and reduction site while remote copper and heme iron centers, magnetically isolated from the site of O_2 binding, are electron acceptors from cytochrome *c* (Hill et al., 1986). By analogy, DβM is proposed to utilize a single copper site for O_2 binding and activation and a remote copper site for shuttling electrons from the exogenous reductant to the site of O_2 activation.

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Registry No. DβM, 9013-38-1; Cu, 7440-50-8; tyramine, 51-67-2.

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[2] In the event that ascorbate bound to the Cu^{2+}–hydroperoxide form of enzyme, ES′, electron transfer would be expected to produce active site hydroxyl radical via Fenton's chemistry (Sheldon & Kochi, 1981). Clearly, according to the mechanism in Scheme III, binding of ascorbate prior to product formation is highly undesirable and may be prevented by structural isomerizations accompanying the formation of ES′ vs EP or simply by the maintenance of a very low steady-state concentration of ES′.

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Kinetic Studies of the Pyridoxal Kinase from Pig Liver: Slow-Binding Inhibition by Adenosine Tetraphosphopyridoxal

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ABSTRACT: Pyridoxal kinase from pig liver has been purified 10 000-fold to apparent homogeneity. The enzyme is a dimer of subunits of M_r 32 000. The enzyme is strongly inhibited by the product pyridoxal 5′-phosphate. Liver pyridoxamine phosphate oxidase, another enzyme involved in the biosynthesis of pyridoxal 5′-phosphate, is also strongly inhibited by this compound [Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089-2095]. Thus, the biosynthesis of pyridoxal 5′-phosphate in the liver might be regulated by the product inhibition of both pyridoxamine phosphate oxidase and pyridoxal kinase. Kinetic studies revealed that the catalytic reaction of liver pyridoxal kinase follows an ordered mechanism in which pyridoxal and ATP bind to the enzyme and ADP and pyridoxal 5′-phosphate are released from the enzyme, in this order. Adenosine tetraphosphopyridoxal was found to be a slow-binding inhibitor of pyridoxal kinase. Pre-steady-state kinetics of the inhibition revealed that the inhibitor and the enzyme form an initial weak complex prior to the formation of a tighter and slowly reversing complex. The overall inhibition constant was 2.4 μM. ATP markedly protects the enzyme against time-dependent inhibition by the inhibitor, whereas another substrate pyridoxal affords no protection. By contrast, adenosine triphosphopyridoxal is not a slow-binding inhibitor of this enzyme.

Pyridoxal kinase (EC 2.7.1.35) catalyzes the transfer of the γ-phosphate moiety of ATP to the 5-hydroxymethyl group of pyridoxal. This enzyme, in conjunction with pyridoxamine phosphate oxidase, is involved in the biosynthesis of PLP[1] (the coenzyme form for vitamin B_6 dependent enzymes) (Snell & Haskell, 1971). It has been reported that liver and brain pyridoxamine phosphate oxidases are strongly inhibited by the product PLP (Wada & Snell, 1961; Kwok & Churchich, 1980). This inhibition may be physiologically important for the regulation of PLP biosynthesis (Snell & Haskell, 1971; Lui et al., 1981). On the other hand, pyridoxal kinase has been purified from the brains of mammalian species (Neary & Diven, 1970; Kwok & Churchich, 1979a; Kerry et al., 1986)

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; AP_4-PL, adenosine tetraphosphopyridoxal; AP_3-PL, adenosine triphosphopyridoxal; AP_4-PN, adenosine tetraphosphopyridoxine.

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