Mechanism of the Insect Enzyme, Tyramine
β-Monoxygenase, Reveals Differences from
the Mammalian Enzyme, Dopamine β-Monoxygenase*

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Tyramine β-monoxygenase (TβM) catalyzes the synthesis of the neurotransmitter, octopamine, in insects. Kinetic and isotope effect studies have been carried out to determine the kinetic mechanism of TβM for comparison with the homologous mammalian enzymes, dopamine β-monoxygenase and peptidylglycine α-hydroxylation monooxygenase. A new and distinctive feature of TβM is its very strong substrate inhibition that is dependent on the level of the co-substrate, O2, and substrate deuteration. This has led to a model in which tyramine can bind to either the Cu(I) or Cu(II) forms of TβM, with substrate inhibition ameliorated at very high ascorbate levels. The rate of ascorbate reduction of the E-Cu(II) form of TβM is also reduced at high tyramine, leading us to propose the existence of a binding site for ascorbate to this class of enzymes. These findings may be relevant to the control of octopamine production in insect cells.

The copper hydroxylases are a unique class of enzymes that are found in eukaryotes and play a critical role in the biosynthesis of neurotransmitters and hormones. The most studied enzymes in this family are peptidylglycine α-hydroxylation monooxygenase (PHM)3 and dopamine β-monoxygenase (DBM) (1). PHM catalyzes the conversion of C-terminal glycine-extended peptides to their α-hydroxylated products, the first step in the amidation of peptide hormones, required for a range of biological activities (2). DBM catalyzes the hydroxylation of dopamine to yield norepinephrine and, thus, is vital for the regulation of these neurotransmitters (3–5). More recently, a third member of this enzyme family was identified, tyramine β-monoxygenase (6). TβM is the insect homolog of DBM, sharing 39% identity and 55% similarity with the mammalian enzyme. TβM similarly catalyzes the hydroxylation of tyramine at the β-carbon position (Scheme 1). Although tyramine plays no role in mammalian physiology, the product of TβM, octopamine, has been shown to act as a neurotransmitter in invertebrates, regulating physiological functions such as neuromuscular transmission, behavioral development, and ovulation (7–9). One advantage to studies with TβM is the much more facile expression system for this enzyme in relation to DBM (10), which makes it possible to pursue structure/function relationships. In this paper, we report a detailed analysis of the kinetic behavior of the wild type TβM, an essential first step in understanding this complex enzyme system.

All three of the copper hydroxylases employ two non-coupled, mononuclear Cu centers for oxygenase activity, termed CuM and CuH. Structural information pertaining to the active site of the enzymes is derived primarily from the crystal structure for PHM and extended X-ray absorption fine structure studies with both DBM and PHM (11–13). Each copper site assumes a unique coordination environment and a distinct mechanistic function. CuM serves as the site of dioxygen binding and activation, whereas the CuH site functions as an electron transfer site in the reaction mechanism (1, 14). The CuM site is coordinated by two histidine residues, a weakly bound methionine, and one or two water molecules, depending on the oxidation state. In the oxidized state, CuH is ligated by three histidine residues and a water molecule in a tetrahedral geometry. Loss of a water molecule at CuH accompanies reduction of the copper center, as for the CuM site. The ligands to both copper centers are fully conserved among all three enzymes. Although little structural information is available for TβM, the sequence and EPR spectra support an active site ligation identical to DBM and PHM (10).

A mechanism for substrate hydroxylation by this class of enzymes has been proposed based on extensive kinetic characterization of DBM and PHM (Scheme 2) (1). Product formation is coupled to the 2e− oxidation of substrate and the 4e− reduction of dioxygen to water, with one atom of O2 incorporated into the final product. The copper atoms supply the two remaining electrons for this process. Ascorbate is required as a co-substrate to regenerate the reduced Cu(I) form of enzyme during turnover, with 2 mol of ascorbate converted to semidehydroascorbate during the one-electron reduction of each Cu(II) site. In the absence of direct evidence for an ascorbate binding site, an outer sphere electron transfer to both CuM and
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Cu_{II} has appeared to be the most likely mechanism for the reductive process. In the oxidative half-reaction, dioxygen reacts with the reduced Cu_{M} center. The initial product of this reaction is believed to be a Cu(II)-superoxide species that subsequently abstracts a hydrogen atom from the bound substrate (15, 16). A long range electron transfer from Cu_{II} to Cu_{M} is required to complete the reaction cycle, leading to formation of the hydroxylated product (17) and reoxidation of both copper centers.

Based on the high sequence similarity and initial reactivity studies (10), the reaction mechanisms of DβM and TβM were predicted to be identical. The detailed kinetic characterization of TβM presented herein, including an examination of the dioxygen and ascorbate dependence in the oxygenase reaction and kinetic isotope effects, reveals several significant differences between the mechanisms of the insect TβM and the mammalian DβM. Substrate inhibition observed in the reaction mechanism of TβM implies tighter regulation of neurotransmitter levels by the insect enzyme. The dependence of the rate of TβM reduction by ascorbate on substrate level suggests, for the first time, a site for reductant binding within this class of enzymes.

EXPERIMENTAL PROCEDURES

Materials—Drosophila S2 cell growth medium was obtained from Invitrogen. Chromatography medium was purchased from GE Healthcare, except Talon affinity resin, which was purchased from BD Biosciences. 4-hydroxy-β,β-[2-2H]phenethyamine hydrochloride (dideuterated tyramine) was synthesized as previously described (18), and the deuterium content was verified by NMR. Catalase was purchased from Roche Applied Science. All other reagents were purchased from Sigma.

Enzyme Preparation and Purification—TβM was expressed in Drosophila Schneider 2 (S2) cells (Invitrogen) according to methods described previously (10). The protein was purified using anion exchange, His tag affinity, and size exclusion chromatography methods as reported (10), with the following modification. The TβM-containing DEAE fractions were dialyzed against 10 mM KP_2, 0.25 mM NaCl, pH 8.0, prior to binding to the Talon resin. Protein concentrations were determined by UV absorbance at 280 nm (ε_{280} = 1.34 ml mg^{-1} cm^{-1}) using a monomer mass of 68,580 Da. Purified TβM (at 3–5 mg/ml) was frozen in 50–100-μl aliquots in liquid nitrogen and stored at −80 °C until further use.

Enzyme Kinetic Assays—The rates of oxygen consumption by TβM were measured on a Yellow Springs Instruments model 5300 biological oxygen electrode. Assay conditions were similar to those described previously in kinetic assays with DβM (19). Reaction mixtures (1 ml) contained 50 mM potassium phosphate (pH 6), 0.1 mM KCl (to maintain ionic strength), 2 μM CuSO_4, and 40 μg/ml catalase, T = 35 °C. Assay mixtures for the measurement of enzyme specific activities contained 250 μM tyramine, 10 mM ascorbate, 0.16 μM TβM, and 222 μM oxygen (air-saturated). An average specific activity for TβM was measured on a Yellow Springs Instruments model 5300 biological oxygen electrode. Assay conditions were similar to those described previously in kinetic assays with DβM (19). Reaction mixtures (1 ml) contained 50 mM potassium phosphate (pH 6), 0.1 mM KCl (to maintain ionic strength), 2 μM CuSO_4, and 40 μg/ml catalase, T = 35 °C. Assay mixtures for the measurement of enzyme specific activities contained 250 μM tyramine, 10 mM ascorbate, 0.16 μM TβM, and 222 μM oxygen (air-saturated). An average specific activity for TβM was determined for each set of experiments and used to standardize data collected over numerous days. The concentration of TβM in the assay solutions varied from 0.1 to 0.4 μM. The concentrations of tyramine (0.025–10 mM), ascorbate (2–200 mM) and dioxygen (11 μM to 1.067 mM) were otherwise varied as indicated under “Results.” The dioxygen concentration in assay solutions was varied by stirring reaction mixtures under a mixed O_2/N_2 atmosphere of the appropriate proportion for ~10 min. The amount of dioxygen in solution was recorded as a voltage by the oxygen electrode and converted to units of molarity based on the known concentration of dissolved oxygen in air-saturated water (222 μM at 35 °C) or O_2-saturated water (1067 μM at 35 °C). All reactions were initiated by the addition of enzyme. Initial velocities of dioxygen consumption by the enzyme were corrected for the ascorbate background rate and fit to the Michaelis-Menten equation, using the program Kaleidagraph. In cases where substrate inhibition was observed, the data were fitted to Equation 1, which includes a term for the inhibition constant, K_i (20).

\[ v = \frac{k_{cat} [E] [S]}{K_m + S + S/K_i} \]  

(Eq 1)
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The values for $k_{\text{cat}}$ and $K_m$ and $K_i$ and errors in these values were derived from the resultant fits. The error in $k_{\text{cat}}/K_m$ or KIE values was obtained through error propagation according to standard methods (21).

RESULTS

Initial kinetic studies indicated that the reaction of TβM with tyramine followed simple Michaelis-Menten kinetics; a $k_{\text{cat}}$ of 1 s$^{-1}$ and an apparent $K_m$ of 161 μM were reported (10). Ferrocyanide was used as the reductant in these experiments. Ferrocyanide is an alternate electron donor for the copper monoxygenases and provides a convenient spectroscopic assay for determining enzyme activities (19). However, ascorbate is the anticipated in vivo reductant as reported for DβM and PHM (2, 19). In the present studies, all kinetics of TβM were pursued using ascorbate as the reductant. This allows the direct comparison of kinetic data obtained with TβM with values previously reported in the DβM and PHM reactions. The use of ascorbate also provides a frame of reference for understanding the physiological properties of TβM.

Initial velocities were measured by the rate of $O_2$ consumption by TβM at varied tyramine (25 μM to 10 mM) and 10 mM ascorbate in air-saturated solutions at 35 °C. From the fit of the data, the apparent $K_m$ for tyramine was determined as 87.6 ± 12.7 μM, comparable with the value reported previously for the ferrocyanide assays (10) and to the $K_m$ obtained in previous activity assays of TβM-containing homogenates from different organisms, using ascorbate (8). The maximum turnover rate under these conditions was calculated as 3.91 ± 0.22 s$^{-1}$, ~4-fold greater than the initial value reported.

A surprising result was the decrease in rates observed at tyramine concentrations of >250 μM (Fig. 1A), indicative of substrate inhibition. This behavior was not evident in the preliminary studies using ferrocyanide; nor has substrate inhibition ever been demonstrated in kinetic studies with the analogous enzymes, DβM or PHM. The fit of the initial rate data to Equation 1 yielded an apparent substrate inhibition constant, $K_{\text{tyr}}$, of 3.5 ± 0.56 mM.

The effect of fumarate on the reaction was also investigated, since fumarate acts as an anion activator in the DβM reaction mechanism (22). Consistent with the initial findings (10), fumarate had no effect, within experimental error, on either $k_{\text{cat}}$, $K_{\text{tyr}},$ or $K_{\text{m,tyr}}$ and was subsequently omitted from all assay solutions.

Ascorbate Dependence—The early kinetic characterization demonstrated a higher substrate specificity for TβM in comparison with DβM (10). This fact and the comparatively low observed $k_{\text{cat}}$ value (DβM = 12.7 ± 0.5 s$^{-1}$ (dopamine); PHM = 39.1 ± 0.5 s$^{-1}$ (hippuric acid) (18, 23)) prompted us to investigate the ascorbate dependence on the TβM reaction rates. Initial rates of oxygen consumption by the enzyme were measured as a function of tyramine, at fixed ascorbate concentrations ranging from 2 to 200 mM (Fig. 1B and Table 1). The rate of turnover, $k_{\text{obs}}$, increases nearly 10-fold in this range, from a value of 1.3 s$^{-1}$ at low ascorbate to a maximum value of 12 s$^{-1}$ at 100 mM ascorbate. The latter value more closely approximates the substrate turnover rates determined for DβM. The effect of increasing ascorbate on $k_{\text{obs}}$ and $K_{\text{m,tyr}}$ results in a minor descending trend in the apparent $k_{\text{cat}}/K_{\text{m,tyr}}$ (Table 1).

However, with the exception of the data at 2 mM ascorbate, $k_{\text{cat}}/K_{\text{m,tyr}}$ appears to be independent of ascorbate concentrations, indicative of a ping-pong mechanism, where ascorbate and tyramine interact with different forms of enzyme, Cu(II) and Cu(I), respectively. The slightly inflated value at 2 mM ascorbate probably reflects the poorer signal/noise ratio of the rate data at such low ascorbate concentrations.

\begin{table}[h]
\centering
\caption{Kinetic parameters for TβM as a function of ascorbate; air-saturated solutions, 50 mM KPP, pH 6, 35 °C.}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Ascorbate} & $k_{\text{obs}}$ & $K_{\text{m,tyr}}$ & $K_{\text{m,tyr}}$ & $k_{\text{cat}}/K_{\text{m,tyr}}(\text{app})$ \\
\hline
2 & 1.31 ± 0.12 & 25.2 ± 6.9 & 2.1 ± 0.8 & 52 ± 15 \\
4 & 3.2 ± 0.2 & 96 ± 12 & 1.3 ± 0.2 & 33 ± 6 \\
10 & 3.91 ± 0.22 & 88 ± 13 & 3.5 ± 0.56 & 45 ± 7 \\
50 & 8.9 ± 0.3 & 235 ± 13 & 4.15 ± 0.41 & 37.8 ± 2.5 \\
100 & 11.9 ± 1.2 & 367 ± 60 & 3.6 ± 1.1 & 32.4 ± 6.2 \\
200 & 8.5 ± 0.5 & 265 ± 36 & 10.3 ± 2 & 31.9 ± 4.7 \\
\hline
\end{tabular}
\end{table}
Noteworthy is the effect of ascorbate on substrate inhibition. Increasing ascorbate attenuates the observed inhibition at high tyramine concentrations (Table 1). The tyramine $K_r$ varies from $\approx 1$–2 mM at low ascorbate to 10 mM at high ascorbate. Since the role of ascorbate is to regenerate the reduced form of the enzyme during turnover, the effect of ascorbate on $K_{r,tyr}$ implies that the inhibitory pathway is minimized when more enzyme is present in the reduced state.

Initial velocity data were obtained in experiments with varied ascorbate (2–200 mM) at fixed tyramine concentrations ranging from 50 to 700 $\mu$M. The resultant fit of the data to the Michaelis-Menten equation provided an estimated limiting $K_m$ for ascorbate of $\approx 16$ mM (Fig. 2 and Table 2). This value represents a lower approximation due to difficulties in attaining levels of saturating tyramine, as a result of the substrate inhibition. However, substrate inhibition is negligible in the chosen range of tyramine concentrations. The inhibitory region is already reached at 1 mM tyramine, regardless of the ascorbate concentration. Therefore, the $K_m$ value obtained from these experiments provides a reasonable estimate.

A small decrease in rates also is observed at high ascorbate concentrations. The kinetic data obtained at 300 and 700 $\mu$M tyramine can be fit easily to the Michaelis-Menten equation. However, at lower tyramine, the data are best fit to Equation 1 containing the additional term for $K_r$. Thus, although elevated ascorbate mitigates the observed inhibition by tyramine, the reductant itself can act as an inhibitor. The inhibition by ascorbate is not very strong, however, with a $K_{r,asc}$ of $>1$ nM even at low tyramine. A more significant trend is evident in the apparent $k_{cat}/K_{m,asc}$ which decreases by a factor of 2.5 in the range of tyramine concentrations examined, showing that high levels of tyramine interfere with the second order reaction between the reductant and enzyme.

$\text{Oxygen Dependence}$—The kinetics of the reaction with tyramine at several fixed dioxygen concentrations in the range of 5–100% $O_2$ subsequently were examined (cf. Fig. 3 for data in the presence of 10 mM ascorbate). These experiments allowed us to ascertain the dependence of the apparent kinetic parameters $k_{cat}, K_{m,tyr}$ and $K_{r,tyr}$ on dioxygen and to establish a $K_m$ for $O_2$. The full range of oxygen-dependent kinetic studies focused on two different ascorbate concentrations, 10 and 50 mM (Table 3), since the dependence of the reaction rates on the reductant concentration already had been established.

The apparent $k_{cat}$ was obtained from the fit of the initial velocity data as a function of tyramine, at each concentration of
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O₂, to Equation 1. A plot of k<sub>cat</sub>(app) versus O₂ (Fig. 4) demonstrates that the near maximal turnover rate is obtained in reactions with O₂-saturated solutions. A value for K<sub>m,O₂</sub> was calculated as ~350 μM (determined from the fit of k<sub>cat</sub>(app) versus O₂ for data collected with tyramine as the substrate) (Fig. 4) and appears to be independent of ascorbate. This value is comparable with the K<sub>m,O₂</sub> obtained for DβM (18). Increasing oxygen results in a regular increase in the apparent k<sub>cat</sub>/K<sub>m,tyr</sub>, consistent with a ternary complex mechanism.

Contrary to the effect of ascorbate, dioxygen amplifies substrate inhibition. K<sub>i,tyr</sub> varies from 8 mM at 5% O₂ (60 μM) to 1 mM at 98% O₂ (850 μM) in reactions with 10 mM ascorbate. A 6-fold reduction in K<sub>i,tyr</sub> is observed in experiments using 50 mM ascorbate. This result is consistent with the premise that elevated ascorbate concentrations diminish tyramine inhibition due to a decrease in the amount of oxidized enzyme present. In contrast to ascorbate, higher oxygen concentrations favor formation of the oxidized, Cu(II) form of the enzyme and result in greater inhibition via formation of the abortive, E-Cu(II)-tyramine complex.

**Kinetic Studies with Tyramine-d<sub>2</sub>—**To obtain further insight into the kinetic mechanism of TβM, initial velocities were measured as a function of dioxygen and deuterated tyramine. A range of deuterated tyramine concentrations (25 μM to 10 mM) and oxygen concentrations (60 μM to ~1040 μM) were analogous to those in experiments with protiated tyramine. Data were collected at 10 and 50 mM ascorbate (Table 4). Replots of the data provided the limiting D<sub>v</sub>k<sub>cat</sub> = 2.58 ± 0.64 (10 mM ascorbate) and 2.75 ± 0.5 (50 mM ascorbate). A value of 1.85 ± 0.5 was calculated for D<sub>y</sub>k<sub>cat</sub>/K<sub>m,tyr</sub> (50 mM ascorbate, 97% O₂). The substrate inhibition made it difficult to obtain D<sub>y</sub>k<sub>cat</sub>/K<sub>m,O₂</sub> with any great accuracy, but values appear to be in the range of values obtained for D<sub>y</sub>k<sub>cat</sub>/K<sub>m,tyr</sub>. The deuterium isotope effect on k<sub>cat</sub> appears somewhat larger than the D<sub>y</sub>k<sub>cat</sub>/K<sub>m</sub> value. These trends are different from DβM, where D<sub>y</sub>k<sub>cat</sub> < D<sub>v</sub>k<sub>cat</sub>/K<sub>m</sub> in the absence of the anionic activator (22).

An isotope effect on K<sub>i,tyr</sub> is evident as well. A decrease in K<sub>i</sub> is observed as a result of increasing oxygen in reaction mixtures containing deuterated tyramine, similar to the effect observed using protiated tyramine. However, the K<sub>i</sub> value for tyramine-d<sub>2</sub> displays a narrower range than K<sub>i,tyr</sub>. Under conditions where dioxygen is increased by ~16-fold (~60 μM to 1 mM), K<sub>i,tyr-d<sub>2</sub></sub> varies from 3 to 2 mM at 10 mM ascorbate and from 7 to 3 mM at 50 mM ascorbate. This represents a smaller decrease in K<sub>i,tyr</sub>-d<sub>2</sub>. Compared with the 6–8-fold decrease in K<sub>i,tyr</sub> observed under analogous conditions. The isotope effect on K<sub>i</sub> can be interpreted in the context of a reduced steady state concentration of the Cu(II) form of enzyme, due to the slower abstraction of deuterium from the labeled substrate and, hence, an elevated level of the E-Cu(II)-tyr-O₂ complex.

**DISCUSSION**

**Oxidative Mechanism—**The TβM oxidative half-reaction seems to proceed in accord with the mechanisms established for the mammalian enzymes, as determined from isotope effect studies. An isotope effect on k<sub>cat</sub> of ~2.7 indicates that hydrogen abstraction from the substrate is partially rate-limiting, as demonstrated for DβM and PHM (23, 24). Values for D<sub>y</sub>k<sub>cat</sub>/K<sub>m,tyr</sub> appear to be virtually independent of ascorbate, which again points to an irreversible step separating reduction of the enzyme and the subsequent chemistry. A plot of the dependence of D<sub>y</sub>k<sub>cat</sub>/K<sub>m,tyr</sub> on dioxygen is shown in Fig. 5 as a compilation of data at 50 mM ascorbate. Although the errors are fairly large, there is a decreasing trend as the O₂ concentration increases from ~60 μM to 1 mM. The fact that D<sub>y</sub>k<sub>cat</sub>/K<sub>m,tyr</sub> remains above unity at the highest oxygen concentration is consistent with an ability of tyramine to dissociate from the E-Cu(II)-tyr-O₂ complex (21). The intercepts of the 1/v versus 1/O₂ reciprocal plots at varied tyramine intersect to the left of the 1/v axis (Fig. 6 for 50 mM ascorbate data), in support of a random mechanism where either tyramine or O₂ can bind first to the reduced enzyme. It would appear that there is no obligatory binding of substrate before O₂ in the TβM reaction. The observed dependence of D<sub>y</sub>k<sub>cat</sub>/K<sub>m,tyr</sub> on O₂ also is consistent with a random mechanism. The full expression for the isotope effect in a random kinetic mechanism is illustrated in Scheme 3 and Equation 2, where k<sub>5H/k<sub>SD = Int</sub></sub> represents the intrinsic isotope effect (25).

### Table 4

| Oxygen | k<sub>cat</sub> | K<sub>m,tyr-d<sub>2</sub></sub> | K<sub>i,tyr-d<sub>2</sub></sub> | k<sub>cat</sub>/K<sub>m</sub> | k<sub>cat</sub>(app) | k<sub>cat</sub>/K<sub>m</sub> (app) |
|--------|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 10 mM ascorbate | | | | | | |
| 56 | 1.14 ± 0.17 | 231 ± 67 | 3.53 ± 1.2 | 4.95 ± 1.6 | | |
| 119 | 1.93 ± 0.15 | 177 ± 29 | 2.8 ± 0.5 | 10.9 ± 2 | | |
| 215 | 2.55 ± 0.26 | 178 ± 36 | 2.1 ± 0.5 | 14.3 ± 3.3 | | |
| 395 | 2.8 ± 0.4 | 145 ± 43 | 2.4 ± 0.8 | 19.4 ± 6.4 | | |
| 587 | 4.08 ± 0.68 | 203 ± 59 | 1.25 ± 0.38 | 20.1 ± 6.7 | | |
| 907 | 3.57 ± 0.63 | 90.6 ± 37.4 | 2.0 ± 0.9 | 39.4 ± 17.7 | | |
| 50 mM ascorbate | | | | | | |
| 63 | 2.0 ± 0.1 | 341 ± 37 | 7.4 ± 1.26 | 5.8 ± 0.7 | | |
| 110 | 2.5 ± 0.2 | 257 ± 43 | 6.0 ± 1.5 | 9.6 ± 1.8 | | |
| 214 | 3.3 ± 0.2 | 245 ± 32 | 7.8 ± 1.7 | 13.5 ± 1.9 | | |
| 416 | 6.37 ± 0.5 | 328 ± 44 | 2.8 ± 0.5 | 19.4 ± 3 | | |
| 598 | 7.8 ± 0.7 | 316 ± 52 | 3.3 ± 0.7 | 24.6 ± 4.6 | | |
| 907 | 6.8 ± 0.7 | 233 ± 47 | 4.0 ± 1.0 | 29.2 ± 6.5 | | |
| 1039 | 7.45 ± 0.7 | 193 ± 37 | 2.86 ± 0.6 | 38.6 ± 8.2 | | |
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The full expression simplifies according to Equations 3 and 4 in the limits of low and high oxygen, respectively.

\[
\frac{(V/K_m)_H}{(V/K_m)_D} = \int \frac{\frac{k_{SH}}{k_4 + k_{d}}}{1 + \frac{k_{SH}}{k_4 + k_{d}}} \quad (\text{Eq. 3})
\]

\[
\frac{(V/K_m)_H}{(V/K_m)_D} = \int \frac{\frac{k_{SH}}{k_4 + k_{d}}}{1 + \frac{k_{SH}}{k_4 + k_{d}}} \quad (\text{Eq. 4})
\]

The larger value for \(k_{cat} / K_m\) in Equation 3 versus \(k_{cat} / k_m\) in Equation 4 leads to the observed decrease in \(\delta k_{cat} / K_m\) in Fig. 5. The oxidative mechanism of TβM is contrary to the oxidative mechanism of PHM, which is equilibrium ordered with respect to binding of substrates (23), and more closely resembles the reaction of DβM with substrates in the absence of anion activators (21).

The isotope effect on \(k_{cat} / K_m\) in the limit of very low oxygen levels remains quite small in relation to previously determined intrinsic isotope effects for DβM and PHM, 10.9 ± 1.9 (dopamine) and 10.6 ± 0.8 (hippuric acid), respectively. This indicates that the barrier for dissociation of bound tyramine is kinetically significant relative to that for C-H abstraction, again consistent with previous studies of DβM and PHM.

Reducive Mechanism and Tyramine Inhibition—Based on the present data, we now describe a mechanism for TβM, which exhibits several notable differences from the mechanisms established for the related mammalian enzymes, DβM and PHM. The most remarkable distinction is the substrate inhibition observed for the reaction of TβM with tyramine. As mentioned earlier, this behavior was not observed in the hydroxylation reactions of either DβM or PHM. The substrates bind solely to the reduced form of the latter enzymes; reaction of the substrate-bound reduced enzyme complex with dioxygen subsequently leads to product formation. In contrast to the mammalian systems, our results support a mechanism in which tyramine also is able to bind to the oxidized form of TβM. The interaction of tyramine with the oxidized form of the enzyme leads to an inhibitory complex, notated as E-Cu(II)-tyr.

The ability of tyramine to form an unproductive complex with the oxidized enzyme explains the effect of ascorbate on substrate inhibition. As the concentration of the reductant is increased, a greater amount of enzyme is present in the reduced form rather than formation of the E-Cu(II)-tyr complex. This is reflected in the large \(K_{I,tyr}\) values observed at elevated ascorbate (Table 1).

The dioxygen dependence and the isotope effect on substrate inhibition further support our conclusions. Contrary to the effect of ascorbate, high dioxygen concentrations increase the steady state level of oxidized enzyme, and formation of the E-Cu(II)-tyr complex becomes more probable. Lower \(K_{I,tyr}\) values, consequently, are observed at high oxygen (Table 3). In the reaction with deuterated tyramine, hydrogen abstraction becomes more rate-limiting, which again increases the steady state amount of reduced enzyme present. Under these conditions, the interaction of tyramine with the oxidized
enzyme is reduced relative to the protio-substrate, leading to a more narrow range of $K_{cys}$ as the oxygen is varied (Table 4). In effect, any condition that alters the steady state amount of reduced or oxidized enzyme is manifest in the substrate inhibition.

Furthermore, it appears that tyramine competes with ascorbate for interaction with the oxidized form of TβM. The notion of two competing pathways available to $E_{ox}$ is corroborated by the effect of substrate on $k_{cat}/K_m$asc (Table 2). In a ping-pong mechanism, as established for DβM and PHM, the values for $k_{cat}/K_m$asc are independent of the substrate concentration. Our data show a tyramine-dependent variation in $k_{cat}/K_m$asc for the reaction of TβM and indicate that tyramine interferes with the function of the reductant. The competition by tyramine may arise from two possible origins; either tyramine binding interferes with the formation of a reduced TβM-ascorbate complex, or bound tyramine reduces the rate of outer sphere electron transfer from ascorbate. Both of these effects are likely to be focused at the CuM site, given the absence of a substrate binding site near CuH in PHM (26). The ability of tyramine to obstruct the reaction of TβM arise from two possible origins; either tyramine binding interaction with the reduced TβM and PHM (12). The differing substrate affinities of the reduced and oxidized TβM suggest further extended conformational changes of the protein scaffold. Despite a high sequence homology between TβM and DβM, the disparate regions of the core may be the key to the observed differences in the reactivity of the enzymes.

The high $K_m$ value for ascorbate ($>16$ mm) seems remarkable, given that considerably lower concentrations of reductant ($<10$ mm) are required to attain maximal turnover rates for both DβM and PHM (28). Ascorbate concentrations in vesicles, such as the chromaffin granules and synaptic vesicles where DβM is localized, can be as high as 20 mm. It is not known definitively whether TβM is expressed in similar vesicles of the invertebrate nervous system. Octopamine, however, is associated with dense core vesicles of dorsal unpaired median neurons in locusts (29, 30), and studies also have shown that TβM is localized in octopaminergic neurons/cell bodies (of honeybees and lobster) (8, 9, 31). A recent study demonstrated that octopamine-containing cells of the Drosophila reproductive system, including vesicles of the ovarian sheath, were immunoreactive for TβM. It is therefore likely that TβM is expressed in vesicles, similar to DβM, consistent with the requirement for high ascorbate concentrations.

The high $K_m$asc observed for TβM is not unprecedented. Two distinct ascorbate $K_m$ values previously were described for DβM (69 μM and 37.2 mm) (28); the affinity of the enzyme for the reductant was highly dependent on pH and the presence of fumarate. Although the results of this earlier study were not fully understood, one interpretation for the varying ascorbate effects involved competition between ascorbate and fumarate for a binding site within the enzyme. Fumarate has no bearing on the reaction of TβM with tyramine, and much higher levels of ascorbate are required to observe activity by the insect enzyme, excluding the possibility of a low secondary $K_m$asc. However, the current evidence suggesting an ascorbate binding site in TβM may warrant a reinvestigation of the role of the reductant in the mammalian systems as well.

Finally, we note that relatively minor changes in the levels of all three substrates (ascorbate, tyramine, and dioxygen) appear to have dramatic effects on the observed rate constant. Recent studies have demonstrated that tyramine and octopamine have antagonistic effects and suggest that behavioral regulation may depend on the balance of these two hormones (7, 32). Thus, TβM may be expected to function under $k_{cat}/K_m$ asc conditions for all three substrates in vivo, making the enzyme exquisitely sensitive to small shifts in cellular conditions. Overall, the TβM kinetics data imply tighter regulation of neurotransmitter levels by the insect enzyme than in the mammalian homologue.
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