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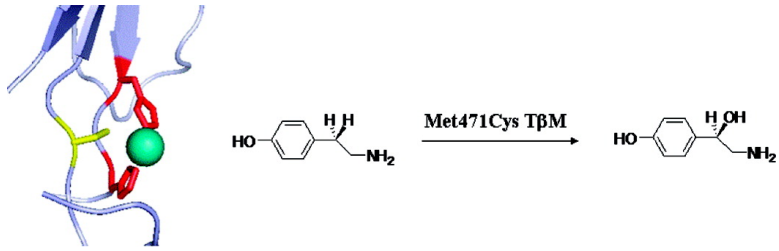

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Hydroxylase Activity of Met471Cys Tyramine β-Monooxygenase

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Abstract: A series of mutations was targeted at the methionine residue, Met471, coordinating the Cu_M site of tyramine β-monooxygenase (TβM). The methionine ligand at Cu_M is believed to be key to dioxygen activation and the hydroxylation chemistry of the copper monooxygenases. The reactivity and copper binding properties of three TβM mutants, Met471Asp, Met471Cys, and Met471His, were examined. All three mutants show similar metal binding affinities to wild type TβM in the oxidized enzyme forms. EPR spectroscopy suggests that the Cu^{II} coordination geometry is identical to that of the WT enzyme. However, substrate hydroxylation was observed for the reaction of tyramine solely with Met471Cys TβM. Met471Cys TβM provides the first example of an active mutant directed at the Cu_M site of this class of hydroxylases. The reactivity and altered kinetics of the Met471Cys mutant further highlight the central role of the methionine residue in the enzyme mechanism. The sole ability of the cysteine residue to support activity among the series of alternate amino acids investigated is relevant to theoretical and biomimetic investigations of dioxygen activation at mononuclear copper centers.

1. Introduction

The neuroregulatory enzymes peptidylglycine α-hydroxylating monooxygenase (PHM), dopamine β-monooxygenase (DβM), and tyramine β-monooxygenase (TβM) are among the few examples of copper-containing oxygenases. These enzymes employ two noncoupled mononuclear Cu centers, Cu_M and Cu_H, to carry out the hydroxylation of their substrates: peptidylglycines, dopamine, and tyramine for PHM, DβM, and TβM, respectively.[1,2] Dioxygen activation, the first step in this mechanism, occurs solely at the Cu_M site; the Cu_H site functions as an electron transfer site, supplying the additional electron required for the oxidation of substrate. Dioxygen binds and reacts with the reduced Cu^{I}_M site to generate what is commonly believed to be a Cu^{II}_M-superoxide species, which subsequently abstracts a hydrogen atom from the bound substrate.[3,4]

The question of how a mononuclear Cu center can effectively catalyze both O_2 and C−H activation has sparked the interests of synthetic chemists as well.[4,5] Several mononuclear copper complexes are known to react with dioxygen to form 1:1 Cu/O_2 adducts, analogous to the reaction at Cu_M.[6] However, these complexes tend to be unreactive toward hydroxylation reactions. The unique coordination environment at the Cu_M site presumably accounts for much of the enzymes' reactivity toward O_2. Two histidines, one to two H_2O/OH molecules, and a weakly bound methionine residue (Met471 in TβM) ligate the tetragonal Cu_M center.[7,8] The methionine sulfur forms a 2.24 Å bond with the reduced Cu site but is absent from the metal coordination sphere in the oxidized enzyme form.[9] Several studies have suggested the importance of this methionine ligand in regulating O_2 binding and the following $Cu^{I}-O_2 \rightleftarrows Cu^{II}-O_2^{-}$ equilibrium;[3,4] the effect of a sulfur group on O_2 binding and dissociation from the metal center also has been illustrated with Cu−β-diketiminate complexes.[10] Furthermore, XAS studies and DFT calculations on PHM have implied that the dynamics of the Cu_M−S bond are critical to the stabilization of postulated Cu_M-O_2 intermediates in the enzymes' catalytic cycle.[4,11]

Studies to directly assess the function of the thioether ligand in the enzymes have been limited, however. The only isolated and well-characterized hydroxylase in which the relevant Cu_M-methionine ligand has been altered is the Met314Ile mutant of PHM.[12,13] Replacement of Met314 by an isoleucine residue in

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Met314Ile PHM resulted in the complete loss of enzyme activity. Significant changes in protein stability and the Cu_H coordination geometry were an additional consequence, further suggesting a structural role for the thioether ligand in the enzyme.[14]

We now have generated a series of mutations directed at the corresponding Cu_M−thioether ligand in TβM, Met471, to probe the importance of the methionine residue for catalysis by the Cu hydroxylases and to determine whether other residues at this site might support activity. TβM shares significant homology with its mammalian counterparts, DβM and PHM, and kinetic studies have demonstrated the mechanisms of the three enzymes to be very similar.[1,15] Given that a high level expression system is unavailable for DβM and that PHM is expressed in mammalian cell lines, the rapid, high-yielding expression of TβM in *Drosophila* S2 cells is ideally suited for mutagenesis studies. Three mutations of the Cu_M−thioether ligand in TβM were investigated: Met471His, Met471Asp, and Met471Cys. The alternate residues at Met471 were expected to modify the redox and/or coordination environment of the Cu_M site and, consequently, affect copper binding and/or dioxygen and substrate activation by the enzyme. DFT calculations for a series of inorganic model complexes have demonstrated the varying effects of anionic and neutral donor groups on dioxygen coordination and activation at mononuclear Cu centers;[16] the alternate residues were chosen with the aim of studying similar effects in the enzyme.

In the present work, the copper binding stoichiometries, EPR spectroscopy, and activities of the TβM mutants were investigated and compared to the wild type enzyme. All three mutants were found to maintain the ability to bind Cu at both sites, and the EPR spectra point to similar coordination geometries for the oxidized enzymes as for the wild type enzyme. However, only the Cys mutant is capable of substrate hydroxylation. The altered kinetics of Met471Cys TβM additionally signify inactivation of the enzyme during the reaction with substrate, further highlighting the singular role of the methionine ligand for enzymatic catalysis. These findings provide the first example of a functional ligand replacement at the conserved methionine in the PHM, DβM, TβM family of copper proteins. The demonstrated importance of a sulfur-containing ligand in catalysis is expected to impact theoretical and biomimetic investigations of dioxygen/C−H activation at mononuclear copper centers.

2. Experimental Section

Materials. *Drosophila* Schneider S2 cells, insect cell growth media, and *Drosophila* Expression System were purchased from Invitrogen. Blasticidin was purchased from Sigma. Primers were custom ordered, HPLC-purified, from Operon. Chromatography media was purchased from GE Healthcare, except Talon affinity resin, which was purchased from BD Biosciences. Assay reagents were purchased from Sigma-Aldrich, except catalase, which was obtained from Roche.

Protein Expression and Purification. Wild type and TβM mutants were expressed as the His-tagged constructs in *Drosophila* S2 cells as described previously.[2] Met471 mutations were generated by PCR using the pBipTBM plasmid and primers encoding ~20 bases upstream and downstream of the mutation. The forward primers for the mutants are shown below, with the mutated codon in bold; reverse primers were complementary.

Met471Asp: 5′-GATTCTCCATCAGCGATGAG**GAT**TGCGTCAACTATATCCAC

Met471Cys: 5′-GATTCTCCATCAGCGATGAG**TGC**TGCGTCAACTATATCCAC

Met471His: 5′-GATTCTCCATCAGCGATGAG**CAC**TGCGTCAACTATATCCACTAC

The resulting altered pBipTBM plasmid was transformed into *Escherichia coli* strain NovaBlue (Novagen) or XL1 Blue (Stratagene) cells and purified using the Qiagen Highspeed Midiprep Kit. The composition of the purified plasmid was confirmed by automated DNA sequencing (University of California, Berkeley, Sequencing Facility and Murdock DNA Sequencing Facility, University of Montana), prior to transfection into Schneider S2 cells.

Cells were harvested, and the protein was purified using anion exchange, His-tag affinity, and size exclusion chromatography as described previously for WT TβM. High purity fractions (single banded, as determined using SDS-PAGE, and comparison to WT enzyme in the case of the mutants) were pooled, and protein concentrations were determined by UV absorbance at 280 nm. TβM molecular weights and extinctions coefficients were determined using ExPASy, on the assumption that all cysteines are half-cystines and neglecting any post-translational glycosylation (http://www.expasy.org). The calculated values for wild type TβM containing the histidine tag are as follows: MW = 69 718 Da, $A_{280nm}^{mg/mL} = 1.423$, $\epsilon_{280} = 99\,210\ M^{-1}\ cm^{-1}$. Concentrations determined by Bradford assays were within 5% of values derived from absorbance at 280 nm.

Enzyme Activity Assays Using Oxygen Electrode. Steady-state rates of oxygen consumption by WT TβM were measured with a YSI model 5300 biological oxygen electrode, and rate constants were calculated as previously described.[15] Assay conditions were identical to those of assay mixtures for HPLC assays.

Product Analysis by HPLC. The HPLC methodology was similar to that previously described for product analysis in the reactions of DβM.[3] HPLC separations were performed on an Alltech Adsorbosphere reversed phase C-18 column. Octopamine and tyramine were monitored at 224 and 274 nm, respectively. Separation of tyramine and octopamine from other assay components was achieved using a mobile phase of 5 mM acetic acid (pH 5.8), 600 μM heptane sulfonic acid, and 13% methanol at 1 mL/min; under these conditions octopamine eluted at a retention time of 5.5 min, and tyramine at 15 min.

Enzyme Activity Assays. Assay solutions containing 500 μM tyramine, 50 mM ascorbate, 50 mM KP_i, 0.1 M KCl, 100−150 μg/mL catalase, and enzyme (0.12−4.4 μM), pH 6, were stirred at 35 °C for periods of 30 min to 13 h, as indicated. $Cu^{II}SO_4$ (2−20 μM) was maintained in all assay mixtures at a ratio of ≥4:1 Cu/TβM, to ensure that the necessary 2 equiv of Cu were bound to the enzyme. Enzyme was added as the final component of the assay mixtures to initiate the reactions. After a specified amount of time the solutions were quenched with 0.1 M $HClO_4$, spun at 10 000 rpm (5 min) to remove precipitated protein, and filtered (0.2 μm syringe filter, Qiagen) prior to injection onto the HPLC column. For experiments monitoring product formation as a function of time, 100 μL aliquots were removed from the reaction mixtures at various time points during the course of the reaction and added to 1 μL of 70% $HClO_4$. The quenched aliquots were similarly spun and filtered prior to HPLC analysis. Assay samples that were not analyzed by HPLC on the same day as prepared were frozen in liquid nitrogen and stored at −80 °C until subsequent analysis.

(13) While CHO cell lines containing Met314Cys and Met314His PHM mutations also have been reported (Kolhekar, A. S.; Keutmann, H. T.; Mains, R. E.; Quon, A. S. W.; Eipper, B. A. *Biochemistry* **1997**, *36*, 10901–10909.), neither of the mutant proteins was isolated and structurally characterized, and activity assays were carried out with spent media rather than using purified protein.

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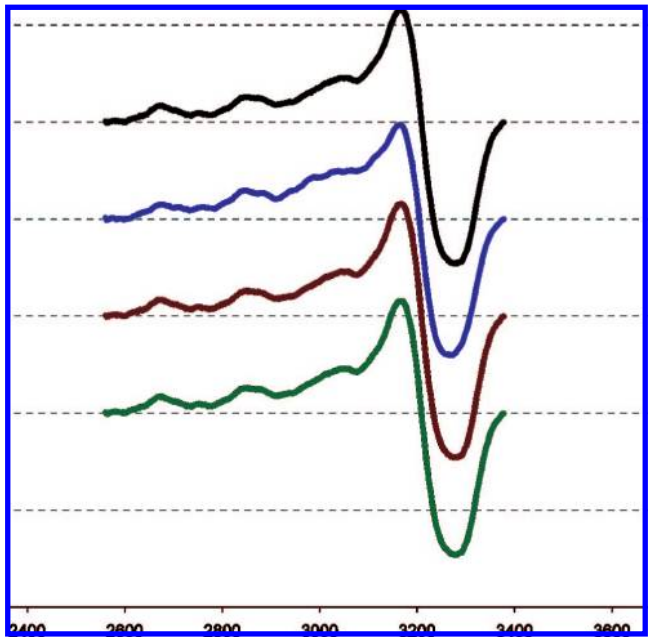


Figure 1. EPR spectra of WT TβM (34.3 μM) (black), Met471Asp TβM (37.5 μM) (blue), Met471His TβM (35.4 μM) (brown), Met471Cys TβM (35.8 μM) (green).

Standard Curves. Although both tyramine and octopamine were monitored by HPLC, tyramine displayed much broader peaks and the integrated area for tyramine was highly dependent on the composition of standard solutions. Octopamine peak areas showed almost no deviation for standard curves generated with a sample in which enzyme, Cu, or ascorbate was left out. Product peaks were very reliable, with sharper absorption peaks and greater accuracy. Therefore, reaction progress was monitored by product formation, while the tyramine absorbance was used only qualitatively as a secondary means to confirm substrate turnover by TβM.

Solutions for the construction of the product standard curve contained varying amounts of octopamine (50−500 μM) and all other assay components except tyramine, including oxidized WT TβM, to eliminate any background absorbance due to enzyme or other reagents. $HClO_4$ (0.1 M) also was added to the standard curve solutions, and the samples were treated similar to the reaction mixtures, as described above. The fit of the resultant standard curve ($y = 196\,256.7x$, $R^2 = 0.9948$) generated from the integrated octopamine peak area (224 nm) was used to quantify the amount of product generated in all reaction mixtures.

The validity of the standard curve was verified on each day of experiments, by measuring at least one octopamine-containing sample of known concentration and comparing the integrated area to the standard curves.

Control Assays. Control reactions, in which solutions containing all reagents except enzyme and varying amounts of Cu (2−20 μM) were stirred for periods of up to 16 h, also were analyzed by HPLC to establish the background amount of tyramine oxidation in the absence of enzyme. Samples were treated in an identical fashion to enzyme assay mixtures, prior to injection onto the HPLC column. A small peak with the same retention time as that of octopamine occasionally was observed in these control assays suggesting that a minor amount of tyramine was oxidized under these conditions, which was somewhat dependent on the copper concentration. However, the maximum amount of octopamine generated in control assays was 22 μM in solutions containing 20 μM Cu^{II}.

The final octopamine concentration in the complete reaction of WT TβM (cf. Figure 2) was 10−20% higher than expected in repeated assays, suggesting the presence of an additional reaction product that absorbed in this region, possibly due to ascorbate reaction products. An additional side product could also account

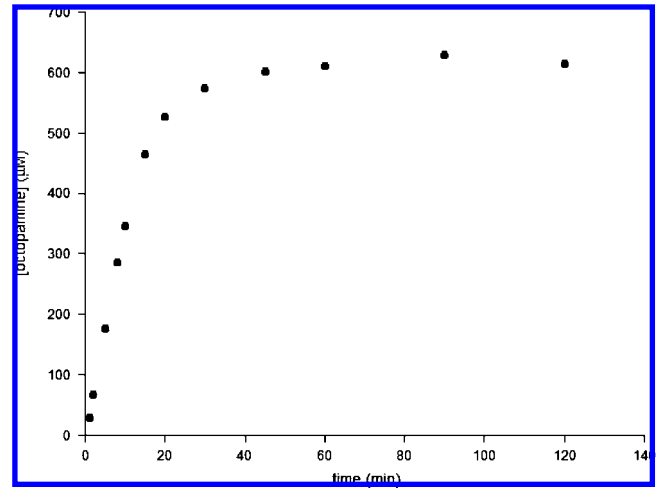


Figure 2. Representative curve illustrating octopamine formation as a function of time in the reaction of WT TβM with tyramine, as determined by HPLC; 50 mM KPi (pH 6), 0.1 M KCl, 50 mM ascorbate, 2 μM $CuSO_4$, 100 μg/mL catalase, 500 μM tyramine, 0.12 μM enzyme; 35 °C.

for the inconsistent, low absorbing peaks occasionally observed in control reactions. However, since the identity of any additional products absorbing in this region is unknown, the maximum absorbance in control reactions was used as the background nonenzymatic tyramine oxidation achievable under the assay conditions, representing an upper limit for octopamine generation in the absence of enzyme.

TβM Samples for EPR. For the purposes of EPR studies, samples of WT TβM and mutant TβM lacking the Histidine-tag were used, since the histidine tag could bind additional metal ions and thereby preclude accurate determination of the protein EPR spectra and the active site Cu-binding stoichiometries. Details for the removal of the His-tag and for the expression and purification of TβM lacking the His-tag will be reported elsewhere.

Preparation of TβM Samples for EPR. TβM samples in 50 mM Tris, 0.1 M NaCl, pH 7.5, were dialyzed against 50 mM Tris, 0.1 M NaCl, 40 μM $Cu^{II}SO_4$, pH 7.5, for 4 h. Dialyzed samples (∼2 mg of TβM, $V = 500$ μL) were diluted ∼10-fold with copper-free Tris buffer, concentrated down to 1 mL (using Millipore Ultrafree centrifugal concentrators, 10 kDa cutoff membrane), diluted to 5 mL once more with copper-free buffer, and concentrated to a final volume of ∼150 μL, to ensure that the unbound Cu content in the enzyme solutions was <5 μM. The samples were spun at 6000 rpm for 2 min to remove any precipitate and transferred to EPR tubes. The final protein concentrations in the EPR samples were 2.1− 3 mg/mL, determined from absorbance at 280 nm. Conversion of mg/mL to μM protein used a MW = 67 757 Da for wild type TβM that was altered appropriately for the mutant enzymes.

Reduced TβM samples were prepared as described above for oxidized TβM samples. However, the samples were sparged with water-saturated argon for ∼30 min and subsequently reduced in an inert-atmosphere glovebox as follows: Ascorbate (slight excess of two molar equivalents) was added to each enzyme sample, and the solutions were spun to remove any precipitate. The reduced samples were transferred to an EPR tube, stoppered, and immediately frozen in liquid nitrogen upon removal from the glovebox.

EPR. EPR spectra were collected using a Varian E9 spectrometer: $T = 15$ K, field center = 2970 gauss, scan range = ± 500 gauss, 9.244 GHz, microwave power = 5 mW, modulation amplitude = 20 gauss, time constant = 0.25 s. Copper concentrations of all enzyme samples were determined by double integration of the resultant EPR spectra using the program EView (E. Bill, Max Planck Institute for Bioinorganic Chemistry) and comparison to the double integration values obtained for the EPR spectra of Cu standards. Cu standards contained 10−150 μM $Cu^{II}SO_4$, 20 mM

Table 1. Cu Content of WT TβM and Met471 Mutants

sample	[TβM] (μM)[a]	[Cu][b]	Cu/TβM
WT TβM	34.3 ± 1.9	62.6 ± 4	1.83 ± 0.16
Met471Asp	37.5 ± 6.5	68 ± 2	1.82 ± 0.33
Met471His	35.4 ± 3.1	68.5 ± 3	1.94 ± 0.19
Met471Cys	35.8 ± 0.3	57.1 ± 3	1.59 ± 0.09

[a] Average of two determinations. Concentrations derived from OD_{280} and the computed MW for each of the mutant enzymes (see Experimental Section). [b] As determined by double integration of EPR spectra, and comparison to double integration values for 75 and 100 μM Cu standards. Errors reflect the uncertainty in the baseline correction used to obtain double integration spectra.

EDTA, pH 8. Several standards in the appropriate concentration range, as well as a cavity spectrum, were measured on each day of EPR experiments.

3. Results and Discussion

Cu Stoichiometry and EPR Spectroscopy. EPR spectroscopy was carried out on oxidized samples (Cu^{II} enzyme form) of WT TβM as well as on the three mutant enzymes, Met471Asp, Met471His, and Met471Cys TβM (Figure 1). The EPR spectra of the three TβM mutants are identical to the WT spectrum, typical of a Type II Cu active site. Therefore, one can conclude that mutation of Met471 does not lead to dramatic changes in the geometry of either metal center in the oxidized enzyme. The results are in agreement with EXAFS studies on the Met314Ile PHM mutant, which also showed that the geometry at the Cu^{II} centers was unperturbed by replacement of the methionine by the isoleucine residue.[9,14]

Double integration of the EPR spectra and comparison to the values obtained for spectra of Cu^{II} standards at similar concentrations, yielded the Cu^{II} content of the copper-reconstituted WT and mutant TβM samples. The ratios of the Cu/enzyme concentrations are indicated in Table 1. Under the conditions for copper reconstitution of the enzyme, a Cu/enzyme ratio of 1.8:1 was obtained for the WT enzyme. The value is just below the expected 2:1 Cu/enzyme ratio but indicates that both active sites are 90% occupied in the sample. Similar results are obtained for the Met471Asp and Met471His TβM samples, which both retain nearly 2 equiv of copper bound per enzyme. The Cu/TβM ratio is slightly lower for Met471Cys TβM (1.5:1, Cu/TβM), which might suggest slightly weaker copper binding at the active site for this mutant enzyme form; however, some variations in copper binding in enzymes from different preparations are common for both DβM and PHM, such that the discrepancy from the wild type Cu/TβM ratio is likely insignificant. The Cu/TβM ratio for the Met471Cys TβM mutant is significantly above 1:1, indicating that both Cu active sites are occupied by the enzyme. It can be concluded that the alternate ligands do not affect binding of Cu^{II} significantly and that the oxidized enzyme forms retain a copper stoichiometry similar to that of wild type TβM.

Following copper reconstitution, samples of WT TβM and the mutant enzymes were reduced with ascorbate to determine whether reduction of both copper centers occurred, as in the case for the wild type enzyme. Reduction with ascorbate resulted in near-complete loss of the EPR signal for WT and mutant TβM samples. Comparison of any remaining signal to the EPR spectrum of a 10 μM Cu standard and to the cavity signal indicated that all four enzyme samples were fully reduced by ascorbate. The reduced TβM EPR samples subsequently were exposed to air and centrifuged to remove any precipitate, and the EPR spectra of the reoxidized samples again were measured. The Cu^{II} signal in the spectra of the reoxidized samples appeared to be almost fully restored (compared with the original spectrum of the oxidized samples, unexposed to ascorbate, at identical concentrations) for all four samples with spectral features virtually identical to the EPR spectra of the oxidized TβM samples.[17] Although, Tris-bound Cu shows significant differences in the EPR from TβM-bound Cu, it was not possible to rule out unequivocally a small loss of Cu^{I} from the enzyme active sites. Based on our results, we conclude that replacement of Met471 with the series of alternate residues, His, Asp, or Cys, does not interfere with the formation of the reduced Cu^{I} form of enzyme by ascorbate. Further spectroscopic studies will be necessary to address the nature of Cu^{I} coordination to the reduced enzyme forms.

Catalytic Behavior. Activity of the Met471 TβM mutants was investigated by monitoring product, octopamine, formation upon reaction of the enzyme with tyramine. Enzyme (0.12−4.4 μM) was added to assay solutions containing 500 μM tyramine, 50 mM ascorbate, 50 mM KP_i (pH 6), 0.1 M KCl, 100−150 μg/mL catalase, and $Cu^{II}SO_4$ at a ratio of ≥4:1 Cu/TβM (to ensure two Cu atoms bound per enzyme). The reaction mixtures were stirred at 35 °C for periods from 30 min to overnight and subsequently quenched with $HClO_4$. The amount of product generated by the enzyme was determined using HPLC methodology, with octopamine separated from the substrate and other assay components on a reversed phase C-18 column. The time-dependence, or final amount of octopamine generated in the assay mixtures, was determined by integration of the product peak area and comparison to standard curve values. Control assays containing all reagents except enzyme and varying amounts of Cu (2−20 μM), with reaction times of up to 16 h, also were analyzed by HPLC to establish the background amount of tyramine oxidation in the absence of TβM. The maximum amount of product generated under these conditions corresponded to 22 μM (with 20 μM Cu^{II}).

As expected, in reaction mixtures containing wild type TβM, tyramine was completely consumed by the enzyme within 15 min under the assay conditions described, with all substrate converted to product (Figure 2). An average value of $k_{cat} = 4.7$ s^{-1} was obtained from the initial slope of the product vs time curve.[18]

Neither Met471His nor Met471Asp TβM displayed any activity with respect to tyramine hydroxylation. HPLC analysis of the final assay solutions of Met471His TβM, with reaction times up to 13 h, did not yield octopamine above the background levels generated in control reactions without enzyme. Similarly, reactions containing 1 μM Met471Asp (5 μM $CuSO_4$) only produced up to 7 μM octopamine, by comparison to standard curve values. This value was not deemed to be significant relative to the background absorbance observed in control reactions. Aliquots from two different enzyme preparations of both Met471Asp and Met471His TβM were examined for

(17) Deviations from the double integration values of original spectrum were within ±25%, with the largest deviation obtained for WT TβM; therefore, the discrepancies likely arise from differences in standard integration values on different days, as well as due to some loss of Cu and/or protein due to handling/freeze−thawing of the samples.

(18) Substrate turnover by WT TβM also was measured independently in a separate assay measuring oxygen consumption by the enzyme. The latter assay provides a more precise measure of initial rates, compared to HPLC analysis. The k_{cat} value obtained for the oxygen electrode assay ($k_{cat} = 3.3$ s^{-1}) under identical conditions is comparable to the value obtained by monitoring octopamine formation by HPLC.

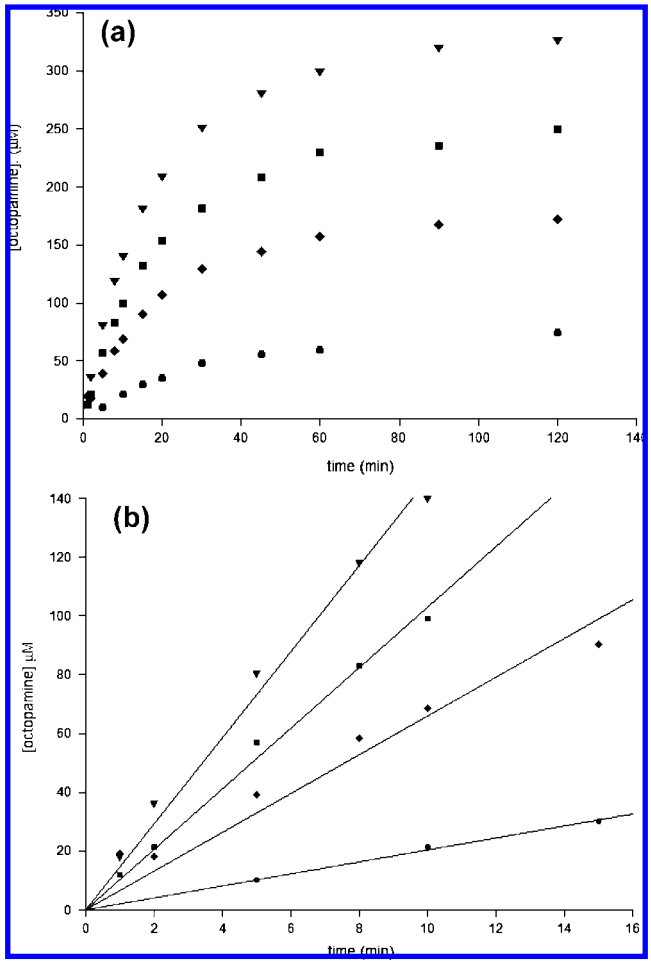


Figure 3. (A) Product vs time curves for the reaction of Met471Cys TβM with tyramine, as determined by HPLC; 50 mM KPi, pH 6, 0.1 M KCl, 50 mM ascorbate, 100 μg/mL catalase, 500 μM tyramine, 35 °C. (●) 1.1 μM Met471Cys, 5 μM Cu^{II}, (◆) 2.1 μM Met471Cys, 10 μM Cu^{II}, (■) 3.2 μM Met471Cys, 15 μM Cu^{II}, (▼) 4.4 μM Met471Cys, 20 μM Cu^{II}. (B) Fit of linear regions of the product vs time curves for the reaction of Met471C TβM with tyramine, at varying [Met471Cys TβM]; 50 mM KPi, pH 6, 0.1 M KCl, 50 mM ascorbate, 100 μg/mL catalase, 500 μM tyramine, 35 °C. (●) 1.1 μM Met471Cys, 5 μM Cu^{II}, (◆) 2.1 μM Met471Cys, 10 μM Cu^{II}, (■) 3.2 μM Met471Cys, 15 μM Cu^{II}, (▼) 4.4 μM Met471Cys, 20 μM Cu^{II}.

activity, since different enzyme preparations often yield variable activities; however, similar results were obtained. It thus appears that neither an aspartate nor a histidine residue in place of Met471 allows for the productive turnover of tyramine by TβM.

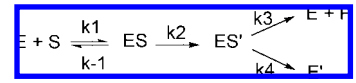
By contrast, octopamine was observed as the product of assays with Met471Cys TβM (Figure 3A). Compared to assays with WT TβM, 10 times the amount of Met471Cys TβM (1.1 μM) was necessary to detect significant amounts of product; the reactions required *ca.* 2 h for completion. The final amount of product formed correlates with enzyme concentration, with a product/enzyme ratio of ~75:1 at all Met471Cys TβM concentrations examined (Figure 3A and Table 2). The results corroborate the enzymatic hydroxylation of tyramine by this mutant. The rate of substrate turnover could be estimated from the initial slopes of the product vs time curves (Figure 3B). The fit of the initial rate data yielded an apparent k_{cat} value of 0.05 s^{-1}, which was fairly consistent for all enzyme concentrations examined, with the exception of the lowest Met471Cys TβM concentration. The observed rate of substrate turnover by

Table 2. Met471Cys TβM Reaction Data

[Met471Cys], μM	$[oct]_f$,[a] μM	$[oct]_f$/[M471C]	$[oct]_f/[tyr]_o$	k_{obs} (s^{-1})[b]
1.09	75	68.8	15%	0.031 ± 0.0005
2.13	170	79.8	34%	0.052[c] ± 0.0031
3.24	250	77.1	50%	0.053 ± 0.0013
4.35	330	75.9	66%	0.056 ± 0.0017

[a] Octopamine concentrations as determined by HPLC. [b] From fit of linear regions of [octopamine] vs time curves, with intercept set to 0. The errors reflect the goodness of fit for the kinetic traces, using protein concentrations derived from OD_{280} and the computed MW for Met471Cys (see Experimental Section). [c] 2.13 μM Met471Cys fit showed greater variance; fit of these data with free-floating intercept gave $k_{obs} = 0.042 \pm 0.0024$ s^{-1}.

Scheme 1. Branching Mechanism in which Met471Cys Forms an Intermediate That Partitions between Product Formation (k_3) and Enzyme Inactivation (k_4)



the altered enzyme is ~80-fold lower in magnitude than k_{obs} for substrate hydroxylation by WT TβM.

In contrast to the WT enzyme, Met471Cys TβM failed to turnover all of the substrate, with only 66% of tyramine converted to octopamine even at the highest Met471Cys TβM concentration examined (4.7 μM). The fact that the ratio of product/enzyme is independent of enzyme concentration is indicative of a secondary reaction process involving inactivation of the mutant enzyme upon reaction with tyramine and dioxygen. The competing processes can be described by Scheme 1, where ES is the enzyme substrate complex, ES′, an intermediate enzyme form, and E′, the inactivated enzyme. The rate constant, k_3, describes the rate of product formation from the ES′ complex, and k_4 is the rate constant for the pathway leading to the inactivated enzyme. The ratio of the two rate constants is described by a partitioning ratio, *r*, which represents the number of catalytic turnovers prior to enzyme inactivation, such that $r = k_3/k_4 = k_{cat}/k_{inact}$.[19,20] The partitioning ratio, *r*, further relates the final amount of product to the initial enzyme concentration by $r = [P]_\infty/[E]_o$. A rate constant can thus be determined for the secondary, inactivation pathway by Met471Cys TβM, based on these relationships. For Met471Cys, the partitioning ratio is given by $[oct]_f/E_o = \sim 75$ (Table 2). This ratio, along with the value determined for $k_{cat}(app) = 0.05$ s^{-1}, yields $k_{inact} = 7 \times 10^{-4}$ s^{-1}. The inactivation of enzyme during turnover contrasts with WT-enzyme, suggesting the importance of a thioether side chain to minimize oxidative damage at a sulfur side chain.[21,22]

Implications for Enzyme Catalysis and Relevance to Model Studies. Of the three mutant TβM forms investigated, it appears that only Cys can replace Met471 at Cu_M and still support substrate hydroxylation. Our findings contrast with recent predictions from theoretical studies describing the effects of various ligand sets on O_2 binding and activation at the Cu_M site of DβM and PHM.[16] Based on their studies, Tolman and co-workers concluded that neutral ligand sets at the Cu_M site

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(such as N_3 or N_2S, where S = Met) would favor Cu^{II}-superoxide formation and render the resultant species more reactive toward H-atom abstraction. Conversely, an anionic ligand at the Cu_M coordination sphere (an OH group in their models) led to increased reduction of the metal-bound dioxygen; $Cu-O_2$ adducts with greater peroxide character have been shown to be less reactive in C−H activation reactions. In accord with these findings, the Met471Asp mutant is unreactive toward tyramine hydroxylation, potentially due to the predicted effect of the anionic ligand on the subsequent dioxygen chemistry. However, the anionic cysteine ligand is able to support activity by Met471Cys and, surprisingly, the neutral N_3 ligand set of the Met471His mutant does not, suggesting that other factors predominate.

Our EPR results indicate that all three mutants are able to bind copper in the oxidized enzyme form, such that a lower affinity for Cu^{II} cannot account for the observed lack of activity for Met471His and Met471Asp. The coordination geometry of the oxidized mutant enzyme forms also appears to be largely unperturbed in comparison to WT TβM, given the similarities among their EPR spectra. However, the exact nature of the coordinated ligands cannot be deduced from simple X-band EPR studies. Changes in bond strengths and distances, particularly for the reduced enzyme form and of subsequent dioxygen adducts, may be significant to the altered activity of the mutant enzymes. While all three alternate ligand sets investigated are expected to result in stable Cu^{I} structures,[16] a lower affinity of the enzymes for the reduced metal cannot be excluded from our data, such that potential metal loss during the catalytic cycle might account for the lack of activity observed for Met471His and Met471Asp TβM. EXAFS spectroscopy should prove valuable in addressing possible changes in the geometry and ligand dynamics at both copper sites.

The sole reactivity of Met471Cys TβM among the three mutants investigated appears to further suggest the importance of a sulfur containing residue for enzyme activity. The inactivation of Met471Cys during turnover, however, suggests that while the methionine residue may not be exclusively necessary to tune the site for reactivity, Met471 may be critical to protect the enzyme against time-dependent damage, most likely due to reactive oxygen species generated during the reaction mechanism. This factor is particularly crucial in light of increasing evidence that the unique reactivity of the Cu hydroxylases stems from their ability to limit the extent of dioxygen activation at the Cu_M site prior to C−H activation.[3,4,16]The effect of the cysteine residue on the various copper−dioxygen intermediates warrants further investigation.

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