

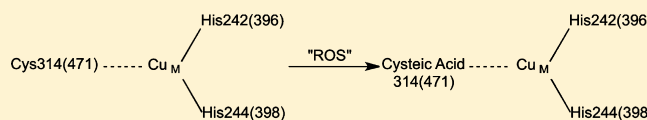
Inactivation of Met471Cys Tyramine β -Monooxygenase Results from Site-Specific Cysteic Acid Formation

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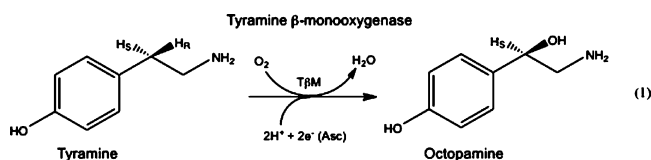
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S Supporting Information

ABSTRACT: Tyramine β -monooxygenase (T β M), the insect homologue of dopamine β -monooxygenase, is a neuro-regulatory enzyme that catalyzes the β -hydroxylation of tyramine to yield octopamine. Mutation of the methionine (Met) ligand to Cu_M of T β M, Met471Cys, yielded a form of T β M that is catalytically active but susceptible to inactivation during turnover [Hess, C. R., Wu, Z., Ng, A., Gray, E. E., McGuirl, M. M., and Klinman, J. P. (2008) *J. Am. Chem. Soc.* 130, 11939–11944]. Further, although the wild-type (WT) enzyme undergoes coordination of Met471 to Cu_M in its reduced form, the generation of Met471Cys almost completely eliminates this interaction [Hess, C. R., Klinman, J. P., and Blackburn, N. J. (2010) *J. Biol. Inorg. Chem.* 15, 1195–1207]. The aim of this study is to identify the chemical consequence of the poor ability of Cys to coordinate Cu_M. We show that Met471Cys T β M is ~5-fold more susceptible to inactivation than the WT enzyme in the presence of the cosubstrate/reductant ascorbate and that this process is not facilitated by the substrate tyramine. The resulting 50-fold smaller ratio for turnover to inactivation in the case of Met471Cys prevents full turnover of the substrate under all conditions examined. Liquid chromatography–tandem mass spectrometry analysis of proteolytic digests of inactivated Met471Cys T β M leads to the identification of cysteic acid at position 471. While both Met and Cys side chains are expected to be similarly subject to oxidative damage in proteins, the enhanced reactivity of Met471Cys toward solution oxidants in T β M is attributed to its weaker interaction with Cu(I)_M.



Tyramine β -monooxygenase (T β M) belongs to a small class of eukaryotic copper-, ascorbate-, and O₂-dependent enzymes that includes dopamine β -monooxygenase (D β M) and peptidylglycine α -hydroxylating monooxygenase (PHM).^{1–3} T β M is the insect homologue of D β M and plays a neuroregulatory role in insects by catalyzing the β -hydroxylation of tyramine to yield octopamine (eq 1).^{4–6}



Studies indicate that T β M expression occurs in octopaminergic neurons.^{5,6} A significant amount of spectroscopic and kinetic data collected for these enzymes has demonstrated a high degree of conservation of mechanism.^{7–14} Structural information about T β M is inferred from primary amino acid sequence homology and the X-ray structures determined for PHM.^{3,15–17} T β M is assumed to use two solvent-exposed, noncoupled mononuclear copper centers, denoted Cu_M and Cu_H and separated by ~11 Å (Figure 1), to incorporate an oxygen atom into the phenethylamine side chain of tyramine^{3,14} (eq 1). O₂ activation and substrate hydroxylation occur exclusively at the Cu_M domain, which is coordinated by two histidines and a Met residue.^{17,18} Cu_H, which is ligated by three

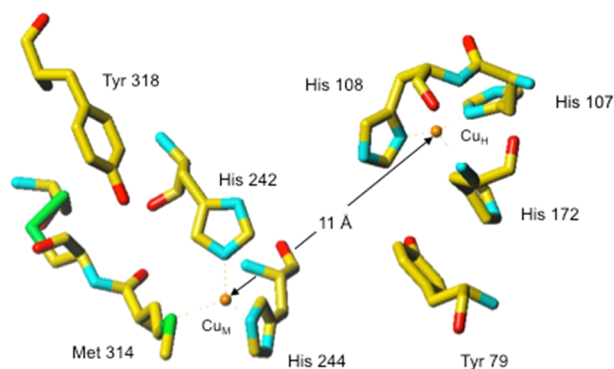


Figure 1. Ligands to Cu_H (His107, His108, and His172) and Cu_M (His242, His244, and Met314) of PHM. Met471 in T β M corresponds to Met314 in PHM (re-created from Protein Data Bank entry 1PHM).¹⁵

histidine residues, serves as an electron reservoir to supply the second electron required for insertion of oxygen into the C–H bond by long-range intramolecular electron transfer.⁷ The combination of structural, kinetic, biochemical, and theoretical evidence supports the formation of a Cu_M–superoxo

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intermediate as the active oxygen species responsible for C–H bond activation at the Cu_M site;^{1,17,19–22} by contrast, the precise timing and pathway for the transfer of an electron from Cu_H to Cu_M remain under active debate.^{19,20,22}

The Met residue at the Cu_M domain appears to be a critical component for catalysis by these enzymes, but the exact function of this residue is not known. The ability of the Cu_M–Met ligand to move into the coordination sphere upon reduction of the copper domains is supported by spectroscopic and crystallographic analyses.^{2,7,9–11,15,23} Extended X-ray absorption fine structure (EXAFS) for the Cu(II) state of PHM, DβM, and TβM indicates an average of 2.5 nitrogen (His) and 1.5 oxygen or nitrogen ligands per copper at 1.97 Å^{2,7,10,11,23} and a weak sulfur ligand detected at 2.71 Å, in agreement with the PHM X-ray crystal structure.¹⁵ Enzyme reduction leads to a shorter Met–copper bond distance: 2.27 Å in PHM, 2.25 Å in DβM, and 2.24 Å in TβM.^{2,10,11,23} Importantly, despite the active site solvent exposure of these enzymes and the requirement for cycling between Cu(I) and Cu(II) during each catalytic cycle, the consumption of O₂ and the generation of hydroxylated product are tightly coupled. Neither the use of a very slow substrate with DβM¹⁹ nor site-specific mutation in PHM (His172Ala),²⁰ each of which diminished *k*_{cat} by nearly 3 orders of magnitude, has any impact on the 1:1 ratio for O₂ uptake and product formation. The aggregate data implicate a dynamic role for the Met ligand to Cu_M that regulates the equilibrium for generating a copper–superoxo species and prevents the diffusional loss of activated oxygen species during turnover.^{1,19,22}

Site-directed mutagenesis has been used extensively to understand the significance of the Met ligand to Cu_M. Studies with PHM in which Met314 was substituted with Ile, His, Asp, and Cys reported that the hydroxylation of glycine-extended peptides was undetectable in spent media,^{2,24} whereas a low activity has been reported for Met314His.²⁵ The X-ray structure of the Met314Ile PHM variant was found to lead to significant disorder at the Cu_H domain, implicating communication between the ligands at distal metal sites.²⁶ Recently, the analogous Met ligand in TβM was mutated to a Cys, His, or Asp, and only the Met471Cys variant retained measurable activity. Electron paramagnetic resonance (EPR) characterization of the Cu(II) forms of WT and Met471 variants has revealed identical properties. Interestingly, Met471Cys TβM was unable to fully convert substrate to product under any conditions examined. This result led to the proposal of a secondary inactivation pathway during turnover.²⁷ XAS/EXAFS experiments were conducted on the series of Met471 variants, to examine how changes in structure relate to the observed altered reactivity, and these showed no binding of His and Asp to Cu(I)_M and only a very weak or small interaction between Cys and the metal site.²⁵ Herein, we demonstrate that the replacement of Met471 with Cys generates an enzyme form that undergoes directed oxidation at position 471. This is attributed to the poor coordination between Cys471 and Cu(I)_M and not to a substrate-dependent inactivation as previously suggested.²⁷

EXPERIMENTAL PROCEDURES

Materials. *Drosophila Schneider* S2 cells, *Drosophila* Expression System, and blasticidin S-HCl were purchased from Invitrogen (Carlsbad, CA). Insect Xpress protein free medium was acquired from Lonza (Basel, Switzerland). Reagents were purchased from Fisher Scientific (Pittsburgh,

PA) or Sigma-Aldrich (St. Louis, MO). Catalase was obtained from Roche. Acetonitrile (Fisher Optima grade, 99.9%) and formic acid (Pierce, 1 mL ampules, >99%) were purchased from Fisher Scientific, and water was purified to a resistivity of 18.2 MΩ cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA). His-tagged Met471Cys TβM was expressed and purified as previously described.^{14,27} Pure fractions (single banded as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis) were pooled, and the concentration was determined by UV absorbance at 280 nm ($\epsilon = 99210 \text{ M}^{-1} \text{ cm}^{-1}$, or $A_{280}^{\text{mg/mL}} = 1.423$).

Product Analysis by High-Pressure Liquid Chromatography (HPLC). The HPLC methodology was similar to that first described for product analysis during reactions catalyzed by Met471Cys TβM.²⁷ Separation of tyramine and octopamine from other assay components was achieved using an Alltech Adsorbosphere reversed phase 10 μm C₁₈ column (Grace Discovery Sciences, Deerfield, IL) (4.6 mm × 250 mm) interfaced to a Beckman-Coulter Gold HPLC system equipped with an autosampler. A mobile phase of 5 mM acetic acid (pH 5.8), 600 μM heptanesulfonic acid, and 15% methanol at a rate of 1.1 mL/min was used to achieve separation of substrate and product. Octopamine and tyramine were monitored at 224 and 274 nm, respectively, and under these conditions, octopamine eluted at a retention time of ~10.5 min and tyramine at ~35 min. Assay mixtures (*V*_T = 1–2 mL) contained tyramine (250 μM to 10 mM), 50 mM ascorbate, 50 mM KP_i, 0.1 M KCl, 100 μg/mL catalase, and enzyme (pH 6). These solutions were mixed for periods of 2 min to 8 h. CuSO₄ was maintained in all assay mixtures at a Cu:TβM ratio of at least 4:1. The reactions were initiated by the addition of enzyme to the assay mixtures. At specific time points, 115 μL aliquots were removed from the assay solution and quenched with 1.5 μL of 70% HClO₄. The aliquots were centrifuged at 14000 rpm (22 min) to remove precipitated protein prior to analysis by HPLC. Samples that could not be immediately analyzed by HPLC were frozen in liquid nitrogen and stored at –80 °C.

Standard Curves. Octopamine standard curves were generated using solutions containing varying amounts of octopamine (50 μM to 2 mM). The amount of product formed during end point and inactivation-based assays was determined by integration of the octopamine peak and comparison to the standards. Generation of a tyramine standard curve and quantification of the loss of substrate were not conducted, because of the broadness of the tyramine peak at 274 nm under the reaction conditions studied. However, the disappearance of tyramine was monitored as a secondary qualitative approach to confirm substrate turnover catalyzed by Met471Cys TβM. The validity of the octopamine standard curve was confirmed with a sample of known octopamine concentration on the same day that reaction samples were analyzed by HPLC.

Enzyme Kinetic Assays Using HPLC. To determine the apparent *K*_m(Tyr) for Met471Cys TβM, end point assays were performed as a function of tyramine concentration. Assay solutions contained a fixed concentration of tyramine (100 μM to 8 mM), 50 mM ascorbate, 100 mM KP_i, 0.1 M KCl, 100 μg/mL catalase, 15 μM CuSO₄, and 3.0 μM enzyme (pH 6) at 35 °C. Enzyme was always added last to initiate the reaction. The rate (*k*_{obs}) at each tyramine concentration was determined from the slope of the line as a result of plotting the amount of octopamine formed at early time points. A background

absorbance, representing a 0.3% contamination of the tyramine stock by octopamine, was subtracted from each time point. The observed rates were first normalized against the published rate for Met471Cys,¹⁴ and the resulting k_{obs} was plotted as a function of tyramine concentration. Initial velocities of octopamine formation were fit to the Michaelis–Menten equation, using Kaleidagraph.

Enzyme Activity–Inactivation Assays. For each assay, $\sim 2.0 \mu\text{M}$ Met471Cys T β M was mixed with 50 mM ascorbate, 100 mM KPi (pH 6.0), 100 mM KCl, 100 $\mu\text{g}/\text{mL}$ catalase, and 10 μM CuSO_4 for 0–4 h at room temperature ($\sim 23^\circ\text{C}$). After incubation of Met471Cys T β M with the reaction components for a precise period of time (0–4 h), 1 mM tyramine was added and reactions were allowed to proceed for up to 4 h. Aliquots (115 μL) were removed at specific time points (2 min to 4 h) during the course of the reaction, added to a 1.5 mL Eppendorf tube containing 1.5 μL of 70% HClO_4 , and centrifuged at 14000 rpm (22 min) to remove precipitated protein prior to injection onto the HPLC column. Samples not analyzed on the same day as the assays were stored at -80°C until the samples could be analyzed by HPLC. These experiments were conducted to quantify the amount of octopamine generated as a function of reaction time, subsequent to enzyme inactivation in the absence of substrate. The rate constant for T β M inactivation was determined by plotting the natural log of the observed rate ($\ln k_{\text{obs}}$) versus the time of incubation prior to the addition of substrate. Control reactions with 2.0 μM WT enzyme were conducted exactly as described for Met471Cys. A WT enzyme preparation with a reduced specific activity allowed the use of identical protein concentrations in the comparison of WT to Met471Cys rates of inactivation.

Control Proteolytic Digest Reactions. Proteolysis of Met471Cys T β M in 100 mM Tris (pH 8.5) was achieved by first denaturing $\sim 15 \mu\text{M}$ enzyme in 8 M urea. All cysteines participating in disulfide bonds were reduced with 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for ~ 20 min at room temperature, and the free cysteines were capped with 10 mM iodoacetic acid (IAA) for ~ 45 min at room temperature. The samples were diluted 4-fold with 100 mM Tris (pH 8.5) to bring the final urea concentration to ~ 2 M, and 1 mM CaCl_2 was added to the solution. One microliter of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Sigma-Aldrich) (0.5 $\mu\text{g}/\mu\text{L}$) was added, and the digestion reaction mixture was incubated in the dark at 37°C overnight. One microliter of neat formic acid was added to each digest reaction mixture before it was analyzed by mass spectrometry.

Proteolytic Digest of Met471Cys T β M Reactions. For each assay, $\sim 15 \mu\text{M}$ Met471Cys T β M was added to a solution containing ascorbate (1 or 50 mM), 50 mM KPi (pH 6.0), 100 mM KCl, 20–100 $\mu\text{g}/\text{mL}$ catalase, 45 μM CuSO_4 , and 0.5–10 mM tyramine for 0–2 h at room temperature. At specific time points, 8 M urea was added to the assay mixture, which was immediately concentrated using a small-volume (0.5 mL) concentrator (YM30, Millipore). This process of denaturing and washing was repeated three times to remove any small molecular reaction components (i.e., KCl, CuSO_4 , ascorbate, tyramine, octopamine), after which Met471Cys T β M was reconstituted in 100 mM Tris (pH 8.5) and concentrated to $\sim 20 \mu\text{L}$. Inactivated Met471Cys T β M was prepared for proteolysis using the same procedure described above for the control reactions.

Liquid Chromatography–Mass Spectrometry (LC–MS). TPCK trypsin-digested samples of Met471Cys T β M

were analyzed with a Waters nanoAcquity ultra-performance liquid chromatograph (UPLC) connected in-line with a quadrupole time-of-flight (Q-ToF) mass spectrometer that was equipped with a nanoelectrospray ionization (nanoESI) source (Q-ToF Premier, Waters, Milford, MA).

Liquid Chromatography. The UPLC was equipped with C_{18} trapping (20 mm \times 180 μm , 5 μm , Waters Symmetry) and analytical (100 mm \times 100 μm , 1.7 μm , Waters BEH130) columns with a 10 μL sample loop. Solvent A consisted of 0.1% formic acid and 99.9% water and solvent B 0.1% formic acid and 99.9% acetonitrile (v/v). Following sample injection, trapping was performed for 8 min with 100% solvent A at a flow rate of 3 $\mu\text{L}/\text{min}$. The elution program consisted of a linear gradient from 25 to 35% solvent B over 30 min, a linear gradient to 55% solvent B over 10 min, a linear gradient to 95% solvent B over 0.33 min, isocratic conditions at 95% solvent B for 6.67 min, a linear gradient to 1% solvent B over 0.33 min, and isocratic conditions at 1% solvent B for 7.67 min, at a flow rate of 500 nL/min. A seal wash period of 5 min was used between sample injections. The injection needle was washed with 500 μL each of solvents A and B to avoid cross-contamination. The analytical column and sample compartment were maintained at 35 and 8°C , respectively.

Mass Spectrometry. The column exit was connected to a Universal NanoFlow Sprayer nanoESI emitter mounted in the nanoflow ion source of the Q-ToF Premier. The nanoESI source parameters were as follows: nanoESI capillary voltage, 2.3 kV; nebulizing gas (nitrogen) pressure, 0.15 mbar; sample cone voltage, 30 V; extraction cone and ion guide voltages, 4 V; and source block temperature, 80°C . No cone gas was used. The collision cell contained argon gas at a pressure of 8×10^{-3} mbar. The ToF analyzer was operated in “V” mode. Under these conditions, a mass resolving power²⁸ of 1.0×10^4 (measured at m/z 771) was routinely achieved, which was sufficient to resolve the isotopic distributions of the singly and multiply charged peptide ions measured in this study. External mass calibration was performed immediately prior to analysis using solutions of sodium formate. Full scan mass spectra were acquired in the positive ion mode over the range of m/z 400–2000, in continuum data format, using a 1.45 s scan integration and a 0.05 s interscan delay.

Tandem Mass Spectrometry (MS/MS). For MS/MS performed in the data-dependent mode, up to five precursor ions occurring within the range of m/z 400–1800 and exceeding an intensity threshold of 30 counts/s were selected from each survey scan for MS/MS. Real-time deisotoping and charge state recognition were used to select +2, +3, +4, +5, and +6 charge state precursor ions for MS/MS. Collision energies for collisionally activated dissociation (CAD) were automatically selected on the basis of the mass and charge state of a given precursor ion. MS/MS spectra were acquired over the range of m/z 50–2000 using a 0.95 s scan integration and a 0.05 s interscan delay. Ions were fragmented to achieve a minimum total ion current (TIC) of 18000 counts/s in the cumulative MS/MS spectrum for a maximum of 3 s. To avoid the occurrence of redundant MS/MS measurements, real-time dynamic exclusion was used to preclude reselection of previously analyzed precursor ions over an exclusion width of $m/z \pm 0.50$ for a period of 180 s. An include list was specified to preferentially select the $[\text{M} + 3\text{H}]^{3+}$ ions of the peptide DDKTAALGGFISISDECCVNYIHYYPATK, which corresponds to residues 456–483 of Met471Cys T β M, in two forms: (1) the form in which both Cys residues are present as

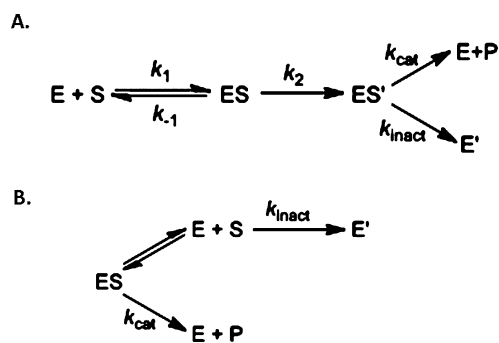
CMC (m/z 1066.47, monoisotopic) and (2) the form in which one Cys residue was present as CMC and the other was cysteic acid (m/z 1063.13, monoisotopic). The inclusion width was $m/z \pm 0.20$.

Mass Spectrometry Data Analysis. The LC–MS/MS data were processed using ProteinLynx Global Server version 2.3 (Waters), which performed background subtraction (threshold 35% and fifth-order polynomial), smoothing (Savitzky–Golay, 10 times, over three channels), and centroiding (top 80% of each peak, and minimum peak width at half-height of four channels) of the mass spectra and MS/MS spectra. The processed data were searched against the amino acid sequence of Met471Cys T β M using the following criteria: nonspecific cleavages, precursor ion mass tolerance of 50 ppm, fragment ion mass tolerance of 0.1 Da, and variable post-translational modifications Met sulfoxide, CMC, cysteic acid, and histidine oxidation. The MS/MS spectra were manually inspected to verify the presence of the fragment ions, which uniquely identify the peptide sequences. The fragment ions were labeled using the nomenclature of Roepstorff and Fohlman.²⁹ Monoisotopic molecular masses are used to identify ions of interest unless stated otherwise.

RESULTS AND DISCUSSION

A series of Met471 T β M mutants was recently generated to probe the role of the Met residue bound to Cu_M. Met471Cys T β M, the only active variant, undergoes a secondary inactivation process during turnover under the conditions examined.²⁷ The fact that the observed octopamine:enzyme ratio is constant (~ 75) at all Met471Cys T β M concentrations examined led to the proposed partitioning of an intermediate form of enzyme (ES') to generate product (E + P) or inactivated enzyme (E') (Scheme 1).²⁷ This analysis allowed

Scheme 1. (A) Initially Proposed Branching Mechanism in Which Partitioning Occurs after Substrate Binding²⁷ and (B) Branching Mechanism in Which the Majority of Enzyme Inactivation Occurs with the Free Enzyme



for the estimation of a rate of inactivation (k_{inact}) of $\sim 7 \times 10^{-4} \text{ s}^{-1}$. While these earlier experiments demonstrated the greater stability of a thioether linkage at position 471, the source and chemical identity of the inactivation process were not pursued.

In this work, the premise of a substrate-mediated inactivation process has been further examined. We first determined the $K_m(\text{Tyr})$ for Met471Cys T β M by measuring the initial rate of octopamine formation via HPLC end point assays as a function of tyramine concentration (Figure S1 of the Supporting Information). The initial rate determined from early time points was plotted versus each tyramine concentration (Figure 2). Surprisingly, the observed K_m of 4.6 (2.0) mM is ~ 20 -fold

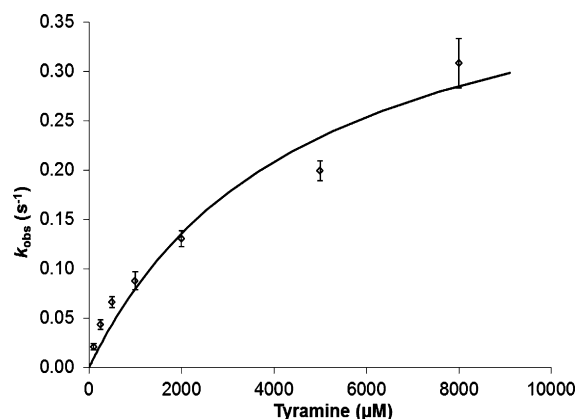


Figure 2. Rates of octopamine formation catalyzed by 3.0 μM Met471Cys T β M at varying concentrations of tyramine in reaction mixtures containing 50 mM KP_i (pH 6), 100 mM KCl, 100 $\mu\text{g}/\text{mL}$ catalase, 50 mM ascorbate, and 15 μM CuSO₄ at 35 °C.

greater than the K_m for the WT enzyme [with the true k_{cat} for Met471Cys, 0.45 (0.09) s^{-1} , now seen to be reduced only ~ 10 -fold in relation to that of WT]. The elevated K_m means that the assays in which the inactivation was initially observed²⁷ were conducted using a starting concentration (500 μM tyramine) that is only 11% of K_m .

Assays were then designed to determine whether solution oxidants alone inactivate Met471Cys T β M. The potential susceptibility of any form of T β M to inactivation by solution oxidants is due to the ability of reduced copper to bind and activate O₂. The susceptibility of the WT forms of D β M and PHM to inactivation by ascorbate has been noted previously.^{30–32} This family of enzymes functions in neurosecretory vesicles, which are enriched with copper, ascorbate, and O₂. Similarly, the in vitro assays require a source of copper (CuSO₄), excess reductant (ascorbate), and O₂ ($\sim 222 \mu\text{M}$ at 35 °C). Met471Cys was incubated with all assay components except tyramine for 0–4 h, followed by the addition of 1 mM tyramine to assay for the percentage of remaining enzyme activity. Met471Cys T β M is essentially inactive after incubation of this form of the enzyme for 2 h in the assay solution before the addition of 1 mM tyramine (Figure S2 of the Supporting Information and Table 1).

Met471Cys T β M was subsequently exposed to the assay mixture in the absence of tyramine for shorter incubation times (0–45 min) to quantify more precisely the rate of inactivation for Met471Cys T β M-catalyzed reactions and to compare the rate of inactivation to that of WT T β M. The slope of the line resulting from the plot of the natural log of k_{obs} , determined by HPLC end point assays (Figure S3 of the Supporting Information), as a function of incubation time provides an estimate for the rate of inactivation ($k_{inact} \sim 3.6 \times 10^{-4} \text{ s}^{-1}$) in relation to the rate for WT of $0.79 \times 10^{-4} \text{ s}^{-1}$ (Figure 3). Replacement of Met471 with a Cys, thus, increases the susceptibility of the enzyme ~ 5 -fold to inactivation. This contrasts with the similar second-order rate constants for reaction of free Cys and Met toward the oxidant hydrogen peroxide at pH 8.^{33,34} While it is difficult to assess the impact of the Cu_M on the protonation state of Cys in Met471Cys, the current incubation conditions of pH 6 are expected to suppress thiolate formation relative to that at pH 8 and, hence, possibly even reduce the intrinsic chemical reactivity for Cys in relation to Met.

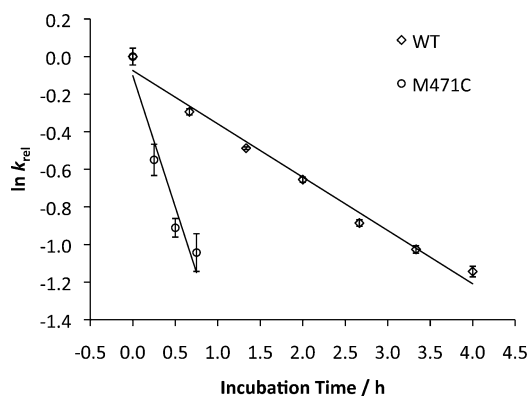


Figure 3. Plot of logarithms of relative initial rates ($\ln k_{rel}$) vs. incubation time, where activities are normalized to 100% at zero incubation time for both traces. For these assays, Met471Cys or WT T β M was incubated at 23 °C with 100 mM KP_i (pH 6.0), 100 mM KCl, 50 mM ascorbate, 10 μ M CuSO₄, and 100 μ g/mL catalase (no tyramine) for various durations before the addition of 1 mM tyramine. Met471Cys T β M was incubated for 0, 15, 30, and 45 min, and WT T β M was incubated for 0, 40, 80, 120, 160, 200, and 240 min. Kinetic assays were executed at 35 °C, and initial rates were determined as slope of formation of octopamine monitored by HPLC vs. reaction time.

In understanding the impact of mutation on the properties of Met471Cys, the key variables are the ratio of turnover (10% of that of WT) to inactivation (500% of that of WT), leading to a 50-fold greater viability in the naturally occurring Met-containing enzyme. We conclude that a Met at position 471 plays a dual role in T β M function: that of controlling the level of the reactive Cu(II)(O₂^{•-}) during catalysis via sulfur ligation (cf. refs 1, 19, and 22), and that of protecting the enzyme against oxidative inactivation. The data in Table 1 indicate

Table 1. Octopamine Formed after Met471Cys T β M Inactivation

incubation time (h) ^a	[octopamine] (μ M) ^b		
	1 h	4 h	6 h
0	163.4	350.3	355.2
2	8.07	17.5	20.1
4	4.02	8.28	11.6

^aThe number of hours all reaction components were allowed to react before the addition of 1 mM tyramine. ^bFor each assay, 2.0 μ M Met471Cys T β M was incubated with buffer [100 mM KP_i (pH 6.0), KCl (100 mM), ascorbate (50 mM), catalase (100 μ g/mL), and CuSO₄ (10 μ M)]. Following the addition of 1 mM tyramine, aliquots (115 μ L) were removed at 5, 10, 15, 30, 60, 120, 240, and 360 min intervals, quenched with 1.5 μ L of 70% HClO₄, and assayed for octopamine formation.

further that the role of substrate in enzyme inactivation is distinct from that originally proposed (Scheme 1A). Independent of the length of incubation of Met471Cys T β M in the absence of tyramine (0–4 h), subsequent addition of substrate leads to an approximate doubling of product formation after an additional 4 h incubation. Of particular note is the fact that a 4 h incubation without substrate virtually eliminates all enzyme activity assayed after 1 h with substrate, whereas incubation of enzyme with substrate for 6 h leads to a substantial level of octopamine production (Table 1). These data indicate a much slower inactivation in the presence of

substrate leading to a mechanism in which the majority of inactivation processes occur with free enzyme (Scheme 1B).

In an effort to identify the residues in Met471Cys modified by solution oxidants, limited proteolysis using TPCK trypsin followed by LC–MS/MS analysis was performed. Numerous tryptic peptides of Met471Cys T β M were analyzed, including several containing active site residues. In particular, the peptide containing Met471Cys T β M (residues 456–483) was observed in two forms: having both Cys residues, at positions 471 and 472, in the form of CMC (m/z 1066.5, +3 charge state) and having one CMC and one cysteic acid (m/z 1063.1, +3 charge state). These peptide ions were well resolved in the precursor ion mass spectrum (Figure 4) and were each individually

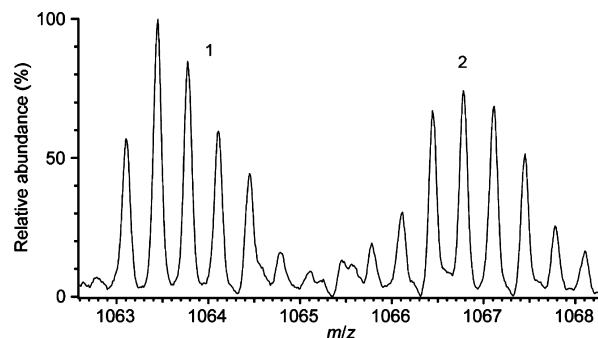


Figure 4. Precursor ion mass spectrum of the $[M + 3H]^{3+}$ ion (m/z 1063.4) of the Cys471-cysteic acid-modified peptide (residues 456–483) (1) and the $[M + 3H]^{3+}$ ion (m/z 1067.7) of the carboxymethyl cysteine (CMC)-modified peptide (residues 456–483) (2).

selected for MS/MS. The fragment ions observed in the MS/MS spectrum for the former precursor ion (m/z 1067.7) identify CMC residues at positions 471 and 472 (Figure 5). Such modification results from reduction and capping of cysteines by TCEP and IAA, respectively, prior to proteolysis. On the other hand, the MS/MS spectrum for the latter

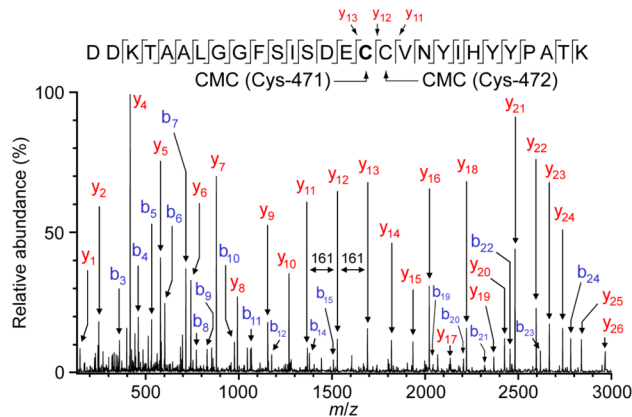


Figure 5. LC–MS/MS analysis of the $[M + 3H]^{3+}$ ion (m/z 1067.7) of the CMC-modified peptide (residues 456–483) from Met471Cys T β M. Met471Cys T β M reactions for proteolytic digest and mass spectral analysis were conducted under the following conditions: Met471Cys T β M (15 μ M), 50 mM KP_i (pH 6), 0.1 M KCl, 50 mM ascorbate, 45 μ M CuSO₄, and 100 μ g/mL catalase. The reactions were quenched with 8 M urea, reduced with 5 mM TCEP, and capped with 10 mM IAA. Proteolysis was initiated with TPCK trypsin (0.5 μ g/mL) and allowed to proceed overnight at 37 °C. Neat formic acid (1 μ L) was added to each reaction mixture before it was analyzed.

precursor ion (m/z 1063.4) identifies a CMC solely at position 472, as indicated by the mass difference between the y_{12}/y_{11} pair fragment ions (161 Da) and the y_{13}/y_{12} pair fragment ions (151 Da). This difference of 10 Da is predicted for cysteic acid at position 471 (Figure 6).

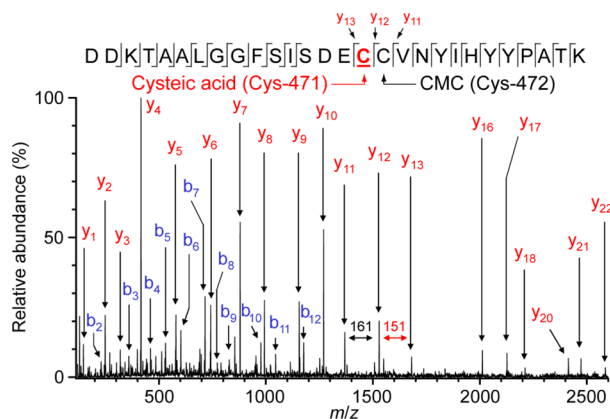


Figure 6. LC-MS/MS analysis of the $[M + 3H]^{3+}$ ion (m/z 1063.4) of the Cys471-cysteic acid-modified peptide (residues 456–483) from Met471Cys $T\beta M$. Conditions were as described in the legend of Figure 5. These data are for the 60 min time point.

It was possible to monitor the fraction of Cys471 in an oxidized, cysteic acid form as a function of time by monitoring the relative mass spectral intensities of the two precursor ions (cf. Figure 4). As summarized in Table 2, the fractions of

Table 2. Fraction of Cysteic Acid for the M471C $T\beta M$ Proteolytic Fragment of Residues 456–483

	0 min ^a	30 min	60 min
50 mM Asc ^b	0.28 ± 0.09	0.66 ± 0.11	0.71 ± 0.07
1 mM Asc	0.28 ± 0.09	0.28 ± 0.05	0.40 ± 0.07
50 mM Asc, 500 μM Tyr ^c	0.28 ± 0.09	0.56 ± 0.05	0.72 ± 0.03

^aFor the 0 min time point, Met471Cys $T\beta M$ (15 μM) was denatured in urea (8 M) and Tris buffer (pH 8.5, 100 mM), reduced with TCEP (5 mM), and capped with IAA (10 mM). Proteolysis was subsequently initiated by TPCK trypsin (0.5 μg/mL) and allowed to proceed overnight at 37 °C. Neat formic acid (1 μL) was added to each reaction mixture before mass spectral analysis. The fraction of cysteic acid detected at 0 min is attributed to the inherent instability at this position during protein purification and workup for the analyses.

^bMet471Cys $T\beta M$ (15 μM) was incubated for 30 and 60 min with the following: KP₁ (pH 6.0, 0.1 M), KCl (0.1 M), ascorbate (50 mM), catalase (20 μg/mL), and CuSO₄ (30 μM). These reaction mixtures were subjected to the same proteolysis conditions as described above.

^cThese are the conditions of the earlier experiment.²⁷

cysteic acid were 0.28 ± 0.09, 0.66 ± 0.11, and 0.71 ± 0.07 at incubation times of 0, 30, and 60 min, respectively, for assay mixtures containing copper and 50 mM ascorbate. The fact that only 71% of the Cys471 was oxidized at the time of 100% inactivation (Figure 3) is attributed to low-level, nonspecific oxidations throughout the rest of the protein.³⁵ Such nonspecific oxidations within tryptic peptides containing amino acid residues inherently susceptible to oxidation (i.e., Met, Cys, and His), but not located at the active site of the enzyme, varied from <1 to 20% and remained relatively constant within experimental error under all conditions examined. Control experiments in which copper was excluded

from the assay solution resulted in nearly identical extents of oxidation compared to those obtained using enzyme without ascorbate and copper. In addition, the extent of oxidation of Cys471 obtained with an assay solution containing a low ascorbate concentration (1 mM) in the presence of copper was almost indistinguishable from control experiments. These measurements support an inactivation process that is primarily the result of time-dependent oxidation of Cys471 by products formed from copper and a high level of ascorbate. As will be discussed in a forthcoming study of mutants of $T\beta M$ at Cu_H (R. L. Osborne et al., unpublished observations), the WT enzyme does not appear susceptible to a targeted oxidation at Met471.

It can be seen that Cys at position 471 results in an unstable hydroxylase that is susceptible to extensive inactivation concomitant with cysteic acid formation (Figure 3 and Table 2). Previously, XAS/EXAFS experiments indicated that replacement of Met471 with a His or Asp eliminates binding to Cu_M in both the oxidized and reduced states of the enzyme, whereas the XAS/EXAFS spectra for Met471Cys are consistent with a very minor binding component of Cys471 to Cu(I)_M.²³ The catalytic activity of Met471Cys $T\beta M$ is ~10% of that of the WT enzyme (Figure 2), and the altered activity can be rationalized by a significantly reduced percentage of enzyme in which Cys471 binds to the Cu(I)_M.²³ The impaired binding of Cys471 to Cu(I)_M and its resulting increased exposure to oxidants also offer an explanation for the impact of Cys replacement on enzyme inactivation. Multiple pathways for Cys oxidation are possible (cf. refs 35 and 36), although a dominant involvement of solution-derived H₂O₂ is unlikely because of the presence of catalase in all incubations and assays. A major unanswered structure–function question concerns the highly specific manner in which Met at Cu_M both stabilizes and activates all members of the $T\beta M/D\beta M/PHM$ family of enzymes.

CONCLUSIONS

The small class of mononuclear dicopper monooxygenases that includes $T\beta M$ has been proposed to function via a catalytic mechanism in which the strength of binding of Met471 to Cu_M limits the extent of superoxide accumulation at this domain prior to substrate activation.²³ The study presented here, which provides a kinetic and chemical rationalization for the previously reported time-dependent inactivation of Met471Cys,²⁷ highlights an additional role for Met471 in stabilizing the enzyme in the presence of oxidants. We propose that it is the poor ability of Cys at position 471 to coordinate to Cu_M in either its oxidized or its reduced state²³ that leaves this residue vulnerable to oxidative processes, resulting in a ratio of k_{cat} to k_{inact} that is reduced 50-fold for Met471Cys relative to that of the WT enzyme.

ASSOCIATED CONTENT

Supporting Information

Graphical analysis of product versus time curves for the reaction of Met471Cys $T\beta M$ at varying concentrations of tyramine, comparative time courses for the stability of Met471Cys $T\beta M$ (with or without tyramine), and raw data for the early time dependence of Met471Cys inactivation (without tyramine). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

T β M, tyramine β -monoxygenase; D β M, dopamine β -monoxygenase; PHM, peptidylglycine α -hydroxylating monoxygenase; XAS, X-ray absorption spectroscopy; EXAFS, extended X-ray absorption fine structure; WT, wild-type; HPLC, high-performance liquid chromatography; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; IAA, iodoacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; UPLC, ultraperformance liquid chromatograph; CMC, carboxymethyl cysteine; CAD, collisionally activated dissociation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Q-Tof, quadrupole time-of-flight; TIC, total ion current.

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