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## Inactivation of peptidylglycine $\alpha$ -hydroxylating monooxygenase by cinnamic acid analogs

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### Abstract

Peptidylglycine  $\alpha$ -amidating monooxygenase (PAM) is a bifunctional enzyme that catalyzes the final reaction in the maturation of  $\alpha$ -amidated peptide hormones. Peptidylglycine  $\alpha$ -hydroxylating monooxygenase (PHM) is the PAM domain responsible for the copper-, ascorbate- and O<sub>2</sub>-dependent hydroxylation of a glycine-extended peptide. Peptidylamidoglycolate lyase is the PAM domain responsible for the Zn(II)-dependent dealkylation of the  $\alpha$ -hydroxyglycine-containing precursor to the final  $\alpha$ -amidated peptide. We report herein that cinnamic acid and cinnamic acid analogs are inhibitors or inactivators of PHM. The inactivation chemistry exhibited by the cinnamates exhibits all the attributes of a suicide-substrate. However, we find no evidence for the formation of an irreversible linkage between cinnamate and PHM in the inactivated enzyme. Our data support the reversible formation of a Michael adduct between an active site nucleophile and cinnamate that leads to inactive enzyme. Our data are of significance given that cinnamates are found in foods, perfumes, cosmetics and pharmaceuticals.

### Keywords

Cinnamate; cinnamate—Cu(I) complex; peptidylglycine  $\alpha$ -hydroxylating monooxygenase; time-dependent enzyme inactivation

### Introduction

Peptidylglycine  $\alpha$ -hydroxylating monooxygenase (PHM) is a copper-, O<sub>2</sub>- and ascorbate-dependent enzyme responsible for the oxidative cleavage of C-terminal glycine-extended precursor peptides to the active  $\alpha$ -amidated peptide and glyoxylate<sup>1,2</sup>. This reaction proceeds by the abstraction of hydrogen from the glycylic C <sub>$\alpha$</sub>  followed by the O<sub>2</sub>-dependent

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### Declaration of interest

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the Louisiana Cancer Research Consortium or the National Institutes of Health.

hydroxylation to generate a C-terminal  $\alpha$ -hydroxyglycine-extended peptide<sup>3,4</sup>. Dealkylation of the  $\alpha$ -hydroxyglycine-extended peptides is catalyzed by peptidylamidoglycolate lyase (PAL), a zinc- and calcium-dependent enzyme (Figure 1)<sup>4-6</sup>. The relationship between PHM and PAL is complex and species-dependent. Vertebrates produce a bifunctional protein, referred to as peptidylglycine  $\alpha$ -amidating mono-oxygenase (PAM), which contains one PHM and one PAL<sup>7</sup>, while invertebrates generate the monofunctional PHM and PAL enzymes from separate genes<sup>8-10</sup>. Bifunctional and pentafunctional (four PHM to one PAL) enzymes are reported from different mollusk species<sup>11,12</sup>. Furthermore, alternative splicing of the PAM mRNA and proteolytic processing of the multifunctional PAM proteins results in a diverse array of catalytically active forms of PAM, PHM, and PAL *in vivo*<sup>13,14</sup>. The significance of the complexities within the PAM/PHM/PAL system is currently unclear, but hints at a regulatory strategy for the production of  $\alpha$ -amidated peptides or toward a biological function for C-terminal  $\alpha$ -hydroxylated peptides.

In mammals, PAM activity has been found in the blood and in many tissues<sup>15</sup>, with the highest levels being found in pituitary, central nervous system and the atrium of the heart<sup>7,14-16</sup>. A large percentage of mammalian bioactive peptides, ~50%, possess an  $\alpha$ -amidated C-terminus. As a consequence, a number of pathological conditions correlate to dysfunction in the expression of specific  $\alpha$ -amidated peptides or one of the PAM/PHM/PAL proteins, including cancer<sup>17,18</sup>, arthritis<sup>19</sup>, inflammation<sup>20,21</sup> and Alzheimer's disease<sup>22</sup>. Peptide amidation is important in insects as well because 80% of all insect bioactive peptides are  $\alpha$ -amidated<sup>23</sup>. Considerable effort has been expended to develop PAM/PHM/PAL inhibitors as such compounds could prove valuable as either insecticides or drugs to treat diseases related to imbalances in  $\alpha$ -amidated peptide production<sup>24-37</sup>. Included amongst these works are reports that cinnamates and ring-substituted cinnamates are irreversible inactivators of PHM<sup>32,33</sup>. Intriguingly, Bradbury et al.<sup>33</sup> suggest that cinnamate-mediated inactivation of PHM results from the formation of a vinyl radical (Figure 1). This chemistry is related to the irreversible inactivation of PHM by *trans*-benzoylacrylate<sup>25</sup>, *trans*-4-phenyl-3-butenolate (PBA, also known as *trans*-styrylacetate)<sup>36,37</sup>, and *trans*-styrylthioacetate<sup>38</sup>; PBA inactivation is proposed to occur via the formation of an allylic radical (Figure 1)<sup>35,37</sup>. The formation of vinyl radical during the cinnamate-mediated inactivation of PHM is mechanistically relevant because this would require the formation of a PAM-based oxidant that is sufficiently strong to abstract a hydrogen atom from the  $\alpha$ -carbon of cinnamate. If true, this may force a revision of the current models for PHM catalysis<sup>39-42</sup>.

In addition to the potential mechanistic significance of the cinnamate-mediated inactivation of PHM, man has a long history of the exposure to and uses for cinnamate and cinnamate derivatives. Cinnamates are found in fruits, fruit juices, vegetables and flowers and are used in perfumes, cosmetics and pharmaceuticals<sup>43-46</sup>. Medically, cinnamates are known to lower blood glucose levels<sup>47</sup> and have potential as treatments for cancer, tuberculosis and other human diseases<sup>48,49</sup>. The inactivation of PHM by cinnamate merits further investigation given the important role of the PAM/PHM/PAL system in the biosynthesis of  $\alpha$ -amidated peptide hormones<sup>1,2</sup>. Herein, we report that cinnamate and a number of cinnamate analogs are PHM inhibitors, information that contributes toward the rational design of PHM-directed therapeutics. A subset of these compounds, cinnamate and three ring-substituted cinnamates,

are also turnover-dependent inactivators of PHM. We find that inactivated PHM is indistinguishable from untreated enzyme and find no evidence for cinnamate oxidation during the inactivation reaction. The reversible Michael addition of an active site nucleophile to cinnamate could account for our data and may explain the inactivation of PHM by a variety of acrylates<sup>20,30,35</sup>, and the 2- and 3-alkenoates<sup>32</sup> as well as provide an explanation for the lack of PHM-labeling by <sup>3</sup>H-PBA<sup>37</sup>.

## Materials and methods

### Chemicals and instrumentation

Cinnamic acid, 2-trifluorocinnamic acid, 3-(3-pyridyl)acrylic acid, phenylpropionic acid, 3,4-methylenedioxy-cinnamic acid, *N,N*-dimethylaminocinnamic acid, urocanic acid, 4-aminocinnamic acid and perdeuterated cinnamic acid (C<sub>6</sub>D<sub>5</sub>-CD=CD-COOH) were from Sigma (St. Louis, MO); bovine catalase was from Worthington (Lakewood, NJ); and [*ring*-<sup>14</sup>C<sub>6</sub>]-*trans*-cinnamic acid (specific activity of 50–60 mCi/mmol and 0.1 mCi/mL) was from American Radiolabeled Chemicals (St. Louis, MO). Recombinant rat bifunctional PAM was a gift from Unigene Laboratories, Inc. (Fairfield, NJ), was greater than 95% pure as judged by SDS polyacrylamide electrophoresis (Figure S1, Supplementary material) and had a specific activity of 4.0 μmol of O<sub>2</sub> consumed/min/mg at 37°C under standard assay conditions<sup>50</sup>. All other reagents were of the highest quality commercially available.

Oxygen consumption was monitored using a Yellow Springs Instrument Model 5300 (Yellow Springs, OH) oxygen monitor. Spectrophotometric studies were performed on a JASCO UV-VIS spectrophotometer (Easton, MD). HPLC separations were performed on an Agilent HP 1100 (Santa Clara, CA), equipped with a four-channel solvent mixing system, a quaternary pump and either a variable wavelength UV detector or a Gilson Model 121 fluorimeter.

### Synthesis of *N*-dansyl-4-aminocinnamic acid

A solution of dansyl chloride (400 mg, 1.5 mmol) in a minimal volume of dry pyridine (~2 mL) was added dropwise to a N<sub>2</sub>-purged solution of 4-aminocinnamic acid (500 mg, 3.1 mmol) in 50 mL of dry pyridine at 60°C. After 24 h, the reaction was diluted and extracted with Et<sub>2</sub>O (100 mL × 3), yielding a yellow oil upon concentration. Crystallization of the final *N*-dansyl-4-aminocinnamic acid was performed with methanol/H<sub>2</sub>O (285 mg, 48%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 8.68 (s, 2H), 8.50 (d, *J* = 8.5 Hz, 3H), 8.31 (d, *J* = 8.5 Hz, 3H), 8.23 (d, *J* = 7.4 Hz, 3H), 7.86–7.81 (m, 1H), 7.63–7.53 (m, 7H), 7.48–7.37 (m, 7H), 7.30 (d, *J* = 8.6 Hz, 7H), 7.17 (d, *J* = 7.6 Hz, 3H), 6.99 (d, *J* = 8.6 Hz, 6H), 6.25 (d, *J* = 16.0 Hz, 3H), 2.85 (s, 21H). *m/z* observed: 397.1. The <sup>1</sup>H NMR spectrum of *N*-dansyl-4-aminocinnamic acid is included in the Supplementary materials (Figure S2, Supplementary materials).

### Measurement of the PHM-dependent consumption of O<sub>2</sub>

Enzyme concentrations were determined by the Bradford method using bovine serum albumin as a standard<sup>51</sup>. The enzymatic reactions were initiated by the addition of PAM (0.5 nmol, 35 μg) into 2.0 mL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol,

0.001% (v/v) Triton X-100, 10 µg/mL bovine catalase, 1.0 µM Cu(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM sodium ascorbate at 37.0 ± 0.1°C. *N*-Acetylglycine (8 mM) served as the oxidizable substrate for the inhibition studies<sup>45</sup>. Initial rates were measured by following the PAM-dependent consumption of O<sub>2</sub> using an oxygen electrode. All kinetic data were determined in duplicate and are reported as mean ± standard deviation.

### PHM inhibition

The initial rates of O<sub>2</sub> consumption by PHM were measured at eight different concentrations of the oxidizable substrate in the absence and presence of three different inhibitor concentrations. *N*-Acetylglycine served as the oxidizable substrate at concentrations of 1.0–45.0 mM. The initial velocities from the inhibition experiments were fit to the equations for competitive, noncompetitive and uncompetitive inhibition, respectively, using SigmaPlot 9.0 (Systat Software, San Jose, CA). All the inhibition data contained herein were the best fit to the one-site competitive inhibition model for the calculation of  $K_{I,obs}$ .

### PHM inactivation

Reactions consisting of 100 µL containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 µg/ml bovine catalase, 1.0 µM Cu(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM sodium ascorbate and the desired concentration of the inactivator were initiated by the addition of 1.0 nmol (70 µg) enzyme and incubated at 37°C. Aliquots of 15 µL were withdrawn at various intervals and diluted into 2.0 mL reactions containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 µg/mL bovine catalase, 1.0 µM Cu(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM sodium ascorbate, and 20 mM *N*-acetylglycine and monitored for O<sub>2</sub> consumption. The inactivator concentrations employed were based on their respective  $K_{I,obs}$  values that had been determined in separate experiments.

### PAL inactivation

Inactivation of PAL activity within bifunctional PAM was evaluated by measuring the initial rates of glyoxylate formation from α-hydroxyhippurate. Reactions of 0.3 nmol (20 µg) of PAM in a 100 µL solution containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, and a concentration of cinnamate or analog equal to 5 × IC<sub>50</sub> were incubated for 2 h. A 20 µL aliquot was removed and used to initiate a 1.0 mL reaction containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, and 10 mM α-hydroxyhippurate. At 10 min intervals from 10 to 60 min, a 100 µL aliquot was removed, added to a vial containing 20 µL of 6% (v/v) trifluoroacetic acid (TFA) to terminate the reaction, and the concentration of glyoxylate measured in the acidified samples.

Glyoxylate was determined by the spectrophotometric method of Christman et al.<sup>52</sup> as modified by Katopodis and May<sup>25</sup>. Standard curves of [glyoxylate] versus A<sub>520</sub> were constructed in the appropriate buffers using a glyoxylate solution that had been calibrated by measuring the glyoxylate-dependent oxidation of NADH (  $\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ) as catalyzed by lactate dehydrogenase.

### Tiopronin protection against the cinnamate-mediated inactivation of PHM

Protection of PHM against the cinnamate-mediated inactivation by tiopronin was evaluated by comparing the rate of glyoxylate formation from *N*-acetylglucine in the absence of cinnamate (no inactivator control), in the presence of cinnamate, and in the presence of cinnamate and tiopronin. Reactions of 0.3 nmol (20  $\mu$ g) of PAM in a 100  $\mu$ L solution containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 0 or 7.5 mM cinnamate, and 0 or 60  $\mu$ M tiopronin were incubated for 2 h. A 20  $\mu$ L aliquot was removed and used to initiate a 1.0 mL reaction containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10  $\mu$ g/mL bovine catalase, 1.0  $\mu$ M Cu(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM sodium ascorbate and 8 mM *N*-acetylglucine and monitored for glyoxylate production. At 10 min intervals from 10 to 90 min, a 100  $\mu$ L aliquot was removed, added to a vial containing 20  $\mu$ L of 6% (v/v) TFA to terminate the reaction, and the concentration of glyoxylate measured in the acidified samples as described above.

### Reversibility of inactivation

To investigate the reversibility of inactivation, a reaction mixture of 250  $\mu$ L containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10  $\mu$ g/mL bovine catalase, 1.0  $\mu$ M Cu(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM sodium ascorbate and 7 mM cinnamate ( $2 \times K_{I,obs}$ ) was initiated by the addition of enzyme and incubated at 37 °C for 2 h. The reaction was then extensively dialyzed and concentrated to 50  $\mu$ L. The concentrated, cinnamate-treated enzyme was then used to initiate a 1.0 mL reaction containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100 and 10 mM hippurate. At various time intervals, the recovery of PHM activity was tested after quenching a 100  $\mu$ L aliquot with 1% (v/v) TFA. The concentration of glyoxylate produced in the acidified samples was used to test for PHM reactivation.

### Investigation of enzyme modification by the cinnamates

Covalent modification of PAM was investigated using *N*-dansyl-4-aminocinnamate, a fluorescent molecule, as an inactivator. A reaction (0.5 mL) containing 100 M MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10  $\mu$ g/mL bovine catalase, 1.0  $\mu$ M Cu(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM sodium ascorbate and 1.0 mM *N*-dansyl-4-aminocinnamate was initiated by the addition of 0.7 nmol (50  $\mu$ g) of enzyme and incubated at 37° C for 3 h. Under these conditions, the mole ratio of *N*-dansyl-4-aminocinnamate/PAM was 710. The reaction mixture was dialyzed against 100 mL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol and 0.001% (v/v) Triton X-100 for 4 h changing the dialysis buffer every hour. The reaction mixture was then concentrated by ultra-filtration to ~100 mL. This sample was then trypsin digested and the peptide fragments were analyzed by reverse phase HPLC (Phenomenex Luna 5  $\mu$  C<sub>18</sub> column, 250 mm  $\times$  4.6 mm) utilizing the in-line fluorescence detector and compared against a standard sample prepared in parallel in the absence of *N*-dansyl-4-aminocinnamate.

Enzyme modification was also examined using [*ring*-<sup>14</sup>C<sub>6</sub>]-*trans*-cinnamate. A 250  $\mu$ L reaction containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10  $\mu$ g/mL bovine catalase, 1.0  $\mu$ M Cu(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM sodium ascorbate



and 3  $\mu\text{Ci}$  (50–60 nmol) of  $^{14}\text{C}$ -cinnamate was initiated by the addition of 0.24–0.70 nmol (18–52  $\mu\text{g}$ ) of PAM. Under these conditions, the mole ratio of [*ring*- $^{14}\text{C}_6$ ]-*trans*-cinnamate/PAM ranged from 70 to 210. The reaction was allowed to incubate at 37° C for 3 h before ultra-filtration was performed. The reaction was then washed with 200  $\mu\text{L}$  of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol and 0.001% (v/v) Triton X-100 and ultra-filtration repeated. The underside of the filtration membrane was then washed with cold poly(ethylene glycol) to remove excess non-enzyme bound radio-labeled cinnamate. Counts per minute were then compared using a scintillation counter to a “no-PAM” control.

Modification of the PAM active site was also investigated as a possible means of cinnamate-mediated inactivation. A 100  $\mu\text{L}$  reaction containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0  $\mu\text{M}$   $\text{Cu}(\text{NO}_3)_2$ , 5.0 mM sodium ascorbate and 3 mM cinnamate was initiated with 0.3 nmol (20  $\mu\text{g}$ ) of PAM and incubated at 37 °C for 12 h. The enzyme was then dialyzed against 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100 for 4 h and the volume reduced to ~40 mL by ultra-filtration. The enzyme sample was analyzed for covalent modification using LC/MS and MALDI-TOF and compared to an enzyme control that lacked cinnamate. The reactions were performed in duplicate and subjected to trypsin digestion. The sample and the no cinnamate control were digested with sequencing grade modified porcine trypsin in 50 mM ammonium bicarbonate buffer (pH 8.2) at a PAM–trypsin ratio of 50:1. Proteolytic digestion was carried out overnight at 37 °C. The resulting peptide mixture were mixed with  $\alpha$ -cyano-4-hydroxycinnamic acid solution (4 mg/mL in 50% (v/v) acetonitrile and 5% (v/v) isopropanol) in 1:1 ratio and spotted onto a blank stainless steel MALDI plate. MALDI-TOF-MS was carried out on 4700 Proteomics Analyzer (Applied Biosystems, Carlsbad, CA) using reflective positive mode. The data were externally calibrated with 4700 Mass Standards (Applied Biosystems, Carlsbad, CA) from the manufacturer.

### Molecular docking studies

Molecular docking studies were performed using the First Discovery 3.0 suite ([www.schrodinger.com](http://www.schrodinger.com)) on a dual 3.2 GHz Xeon workstation running Fedora Core 4 to investigate the possible binding modes of the inhibitors. The crystal structure for reduced PHM<sup>53</sup> was obtained from the Protein Data Bank (<http://www.rcsb.org/pdb/>, 1SDW). All co-crystallized species determined to be redundant for ligand binding were removed (nickel, water, glycerol and substrate). Formal charges for enzyme-bound copper ions and bond orders were corrected, and hydrogen atoms were added using Maestro. Further receptor refinements were carried out utilizing ProteinPrep from within Maestro. Investigation of inhibitor binding modes was performed using Glide<sup>54</sup> and Qsite<sup>55</sup> jointly for quantum-polarized ligand docking. This method generates highly accurate binding modes by quantum mechanically calculating the partial atomic charges of the docked ligand using B3LYP/6-31G\* within the receptor and, subsequently, re-docking the ligand<sup>56,57</sup>. All default settings were used.

### Density functional theory investigation of bond dissociation energies

The relative bond dissociation energies of  $\text{C}_\alpha\text{-H}$  for select cinnamic acid analogs were calculated using Jaguar<sup>58</sup> to reveal any correlation between these values and the inactivation

kinetic parameters. Density functional theory calculations were performed with the B3LYP hybrid exchange-correlation functional and the 6-31G\* basis set. The geometries were optimized for all molecules in the gas phase. The C<sub>α</sub>-H bond was then increased from 1.08 to 3.0 Å with a step-size of 0.1 Å, while holding the molecule rigid, and single point energies were calculated.

## Results

### PAM inhibition by cinnamic acid analogs

Cinnamate and several cinnamate analogs were initially investigated as reversible PHM inhibitors. Cinnamate competitively inhibited the PHM-dependent consumption of O<sub>2</sub>, using *N*-acetyl glycine as the oxidizable substrate, as shown in Figure 2. Steady-state inhibition constants for cinnamate and the cinnamate analogs are included in Table 1. The observed inhibition constants,  $K_{I,obs}$ , ranged by a factor of 1000 from 10 μM for *N*-dansyl-4-aminocinnamate to 10 mM for urocanic acid. The relatively tight-binding of *N*-dansyl-4-aminocinnamate to PHM is consistent with earlier structure–activity studies indicating that peptide substrates possessing a hydrophobic amino acid in the penultimate position have the highest (V/K)<sub>app</sub> values<sup>59</sup>.

### PHM-specific inactivation by cinnamate and cinnamate analogs

Cinnamate and the cinnamate analogs were first evaluated as PHM inactivators by the dilution method, a common method for determining the kinetic parameters for time-dependent inactivators<sup>60</sup>. In agreement with the earlier report by Bradbury et al.<sup>33</sup>, we found cinnamate to inactivate the PHM-catalyzed consumption of O<sub>2</sub> in the presence of *N*-acetyl glycine and ascorbate (Figure 3A).<sup>\*</sup> The 1/ $k_{inact,obs}$  versus 1/[cinnamate] replot was linear (Figure 3B)<sup>56</sup>.<sup>†</sup> In addition to cinnamate, only three other compounds, *N*-dansyl-4-aminocinnamate, 4-aminocinnamate and *N,N*-dimethyl-4-aminocinnamate were found to inactivate PHM (Table 2). No deuterium isotope effect was observed on the cinnamate-mediated inactivation as the ( $k_{inact}/K_I$ )<sub>obs</sub> value obtained using perdeuterated cinnamate was identical to that for cinnamate within experimental error (Table 2). The steady-state inhibition constant,  $K_{I,obs}$ , for cinnamate, perdeuterated cinnamate, *N*-dansyl-4-aminocinnamate, 4-aminocinnamate, and *N,N*-dimethyl-4-aminocinnamate (Table 1), was measured under conditions (relatively short-time frames) such that the rate of the PHM inactivation contributed little to the observed inhibition.

PHM inactivation by cinnamate (Table 2) was pseudo first order and was dependent upon the initial concentration of the inactivator, O<sub>2</sub>, and ascorbate (Figure 3B and Figure S3, Supplementary materials). In addition, tiopronin, a PHM substrate<sup>61</sup>, did protect PHM from inactivation by the cinnamate-mediated inactivation (Figure S4, Supplementary materials).

<sup>\*</sup> A reviewer noted that the no cinnamate control in Figure 3(A) exhibited loss of activity. A repeat of the experiment to eliminate the activity loss in the no cinnamate control is difficult to accomplish because PHM exhibits a loss of activity under turnover conditions, probably the result of reactive, reduced O<sub>2</sub>-species that form during catalysis (see reference<sup>77</sup>). The inclusion of catalase in the inactivation experiments serves to decrease this problem as much as possible.

<sup>†</sup> The inactivation and inhibition parameters were measured at one fixed concentration of O<sub>2</sub> and ascorbate and are, thus, reported as the  $k_{inact,obs}$ ,  $K_{I,obs}$ , and ( $k_{inact}/K_I$ )<sub>obs</sub>.



The enzyme used for our experiments was bifunctional rat PAM comprised the separate and catalytically active PHM and PAL domains<sup>50</sup>. Consumption of O<sub>2</sub> in the presence of ascorbate and *N*-acetylglycine represents the reaction catalyzed by PHM; thus, inactivation of O<sub>2</sub> consumption by the cinnamates only reports on the inactivation of the PHM domain of bifunctional PAM. The cinnamate and a number of cinnamate analogs were tested as PAL inactivators to investigate whether PAM inactivation was specific to the PHM domain. The data indicate that PAL is unaffected by the various cinnamates tested and retained 100% activity when compared to the control reaction (Figure S5, Supplementary materials). Extensive dialysis of bifunctional PAM incubated with cinnamate yielded inactive enzyme unable to catalyze the production of glyoxylate from hippuric acid. All attempts to generate cinnamate-labeled enzyme were unsuccessful. MALDI-TOF analysis of cinnamate-inactivated PAM showed no differences between the treated enzyme and the untreated control (Figure 4). Incubation of PAM with <sup>14</sup>C-cinnamate in the presence of O<sub>2</sub> and ascorbate yielded unlabeled protein, again devoid of PHM activity. Similarly, the incubation of bifunctional PAM with *N*-dansyl-4-aminocinnamate, in the presence of O<sub>2</sub> and ascorbate, yielded enzyme devoid of PHM activity and also lacking any fluorescent label.

Key to fully understanding the impact of our labeling experiments is (a) the limit of detection of fluorescently labeled enzyme potentially generated by inactivation with *N*-dansyl-4-aminocinnamate and (b) the limit of detection of <sup>14</sup>C-labeled enzyme potentially generated by inactivation with <sup>14</sup>C-cinnamate. Our level of detection of *N*-dansyl-4-aminocinnamate is 20 pmol. We used 700 pmol of enzyme in our experiments to identify fluorescently labeled peptides from PHM inactivated with *N*-dansyl-4-aminocinnamate. We applied the tryptic digest of the *N*-dansyl-4-aminocinnamate inactivated PHM directly to the RP-HPLC column to identify any labeled peptides using our in-line fluorescent detector and no fluorescently labeled peptides were found. Assuming that our peptide losses are low and that a 1:1 cinnamate–PHM adduct forms, we would expect ~700 pmol of fluorescently labeled peptide, which is ~35-fold above our level of detection. These experiments show that labeled PHM cannot be a major product of the *N*-dansyl-4-aminocinnamate inactivation chemistry. However, a very low extent of labeling, an <0.05:1 cinnamate–PHM adduct, would be below our level of detection. The background level for the <sup>14</sup>C labeling experiment was 20–30 cpm. We incubated 0.24–0.70 nmol of PHM with 50–60 nmol of [*ring*-<sup>14</sup>C<sub>6</sub>]-cinnamate, removed the unreacted <sup>14</sup>C-cinnamate after the inactivation reaction by ultrafiltration, and found no <sup>14</sup>C-label in PHM above background. Again, assuming a low loss of PHM and formation of a 1:1 <sup>14</sup>C-cinnamate–PHM adduct, we expected to generate 0.24–0.70 nmol of <sup>14</sup>C-labeled PHM possessing 10–40 nCi of radioactivity based on specific radioactivity of the <sup>14</sup>C-cinnamate (50–60 mCi/mmol). With a quenching efficiency of 60–80% for <sup>14</sup>C, we would have found <sup>14</sup>C-labeled PHM in the range of 13 000–70 000 cpm – far above background. Only a very low extent of <sup>14</sup>C-labeling, an <0.001:1 cinnamate–PHM adduct, would be below our level of detection. The most reasonable conclusion based on our *inability* to find labeled PHM upon the inactivation with *N*-dansyl-4-aminocinnamate or [*ring*-<sup>14</sup>C<sub>6</sub>]-cinnamate is that no labeled enzyme is produced. A result consistent with our analysis of inactivated PHM by mass spectrometry and that obtained for the incubation of PHM with <sup>3</sup>H-PBA<sup>37</sup>.

## Computational chemistry and modeling

*In silico* docking of cinnamate and the cinnamate analogs indicated that all bind in the PHM active site consistent with the competitive inhibition observed for cinnamate (Figure 2). The docking pose for cinnamate is shown in Figure 5(A) and the position of cinnamate in the PHM active site relative to bound substrate is shown in Figure 5(B). All of the compounds form a salt bridge between the carboxy terminus and the guanidino group of R240 similar to that observed for the glycine-extended substrates<sup>53,62</sup>. This interaction aligns the C $\alpha$ -H in close proximity to Cu<sub>M</sub>, one of the two copper atoms bound to PHM<sup>53,62</sup>. However, the lack of glycyl amide hydrogen bonding with the N316 coupled with the relatively small size of the compounds prevents interaction with the nearby hydrophobic pocket, suggesting greater mobility when compared to glycine-extended peptide substrates.

## Discussion

### Structure–activity data

Cinnamate is structurally related to two PHM substrates, *N*-cinnamoylglycine and *N*-hydrocinnamoylglycine<sup>24</sup>, and two PHM inactivators, PBA and *trans*-benzoylacrylate<sup>25,34,36,37</sup>. These compounds bind to PHM with higher affinity than cinnamate ( $K_I = 4$  mM):  $K_{M,\text{hydrocinnamoylglycine}} = 0.9$  mM,  $K_{M,\text{cinnamoylglycine}} = 0.2$  mM,  $K_{I,\text{PBA}} = 0.05$  mM and  $K_{I,\text{benzoylacrylate}} = 0.2$  mM, at least to the extent that  $K_M$  and  $K_I$  values reflect relative binding affinities to PHM. Our modeling results indicate that the carboxylate moiety of cinnamate interacts with R240 in the PHM active site (Figure 5), as was demonstrated for the C-terminus of the peptide substrates<sup>62</sup>. The PHM active site possesses a hydrophobic pocket<sup>53,63</sup> and our modeling results indicate that the phenyl moiety of cinnamate cannot reach from R240 to this hydrophobic pocket (Figure 5A). The presence of a longer spacer between the carboxylate moiety and the phenyl ring, like that found in *N*-cinnamoylglycine, *N*-hydrocinnamoylglycine, PBA, and *trans*-benzoylacrylate, enables the phenyl moiety to interact with the hydrophobic pocket within the PHM active site and, probably, is the main reason these four compounds are bound to PHM with higher affinity than cinnamate (Table 1). The relatively tight binding of *N*-dansyl-4-aminocinnamate to PHM may also result from the interaction of *N,N*-dimethyl-1-naphthylamine moiety of the dansyl group with the hydrophobic pocket of PHM. The small difference in the  $K_I$  values of cinnamate and phenylpropionic acid is likely a result of the differential positioning of the phenyl ring within the PHM active site. Cinnamate analogs with a replacement of the phenyl ring with either a pyridine ring (3-(3-pyridyl)acrylate) or an imidazole ring (urocanate) bind to PHM with lower affinity. The pyridine and imidazole rings must interact less favorably with amino acids within the PHM active site, leading to higher  $K_I$  values for 3-(3-pyridyl)acrylate and urocanate relative to cinnamate.

Decoration of the phenyl ring of cinnamate with substituent groups results in changes in the affinity of PHM for the cinnamate derivative. 2-Trifluoromethyl-, 3,4-methylenedioxy-, 4-amino-, and 4-nitro-cinnamate all bind to PHM with greater affinity than cinnamate and *N,N*-dimethyl-4-aminocinnamate binds to PHM with approximately the same affinity as cinnamate (Table 1). The differences in PHM affinity observed for this set of compounds must be related to differences in interactions between the substituent group decorating the

phenyl ring and active site amino acid(s). More definitive SAR results are not possible given the limited set of readily available ring-substituted cinnamates.

### Cinnamate-mediated inactivation

Cinnamate, *trans*-benzoylacrylate, and PBA are not the only olefinic compounds known to inactivate PHM. Other olefinic inactivators of PHM include monoethyl fumarate<sup>25</sup>, styrylthioacetate<sup>38</sup>, 2- and 3-alkenoates<sup>32</sup>, vinylglycine<sup>32</sup>, 3-substituted acrylates<sup>32</sup>, substituted 4-oxo-2-hexenoates<sup>30</sup>, and D-Phe-L-Phe-L-vinylglycine<sup>64</sup>. Other monooxygenases, P<sub>450</sub> and dopamine  $\beta$ -monooxygenase (D $\beta$ M, structurally and mechanistically related to PHM), are inactivated by olefins<sup>65–68</sup>. Most of these olefinic inactivators exhibit characteristics of suicide substrates: the inactivation only occurs during enzyme turnover and substrates or competitive inhibitors protect the monooxygenases against inactivation. In addition, olefinic inactivation of PHM, D $\beta$ M, and P<sub>450</sub> is not mediated by the initial formation of the corresponding epoxide<sup>37,65,67</sup>. The inactivation of D $\beta$ M and P<sub>450</sub> leads to both the generation of olefin-derived oxidation products and to the labeling of the inactivated enzyme. Padgett et al.<sup>67</sup> reported a partition ratio,  $k_{\text{cat}}/k_{\text{inact}}$ , of 900 for inactivation of D $\beta$ M with phenyl(aminomethyl)ethane and found that inactivation with [<sup>3</sup>H]-phenyl(aminomethyl)ethane yields <sup>3</sup>H-labeled DbM. The inactivation of P<sub>450</sub> with 2-isopropyl-4-pentenamide exhibited a partition ratio of 200–300 and led to the alkylation of the enzyme-bound heme<sup>66</sup>. The olefinic inactivation of D $\beta$ M and P<sub>450</sub> was proposed to result from the formation of an olefin-derived radical intermediate<sup>66,67</sup>.

The chemistry for the inactivation of PHM by the cinnamates must be different. Incubation of PHM with PBA results in both O<sub>2</sub> consumption and the generation of oxidized products, 2- and 4-hydroxy-4-phenyl-3-butenate, with a partition ratio of 85–90<sup>37</sup>. None of the compounds tested within this study showed any O<sub>2</sub> consumption (above background) when screened for activity even though inactivation was O<sub>2</sub>- and ascorbate-dependent (Figure S3, Supplementary materials). A low background rate of O<sub>2</sub> consumption is observed for PHM in the absence of an oxidizable substrate resulting from the presence of Cu(II) and ascorbate in an aerobic solution<sup>69</sup>. Incubation of *N*-dansyl-4-aminocinnamate with PHM in the presence of ascorbate and O<sub>2</sub> does not result in any change in C<sub>18</sub>-reverse phase HPLC retention time for *N*-dansyl-4-aminocinnamate; additional evidence that the cinnamates are not PHM substrates. Finally, the lack of a deuterium kinetic isotope effect when using perdeuterated cinnamate as an inactivator on either  $k_{\text{inact,obs}}$  or  $(k_{\text{inact}}/K_{\text{i}})_{\text{obs}}$  indicates that cinnamate-mediated inactivation of PHM does not involve rate-determining C–H bond cleavage.

Incubation of PHM with either [<sup>14</sup>C]-cinnamate or *N*-dansyl-4-aminocinnamate yielded inactive enzyme that was not labeled. Our comparison of trypsin-digested cinnamate-inactivated enzyme to control (untreated) enzyme by MALDI-TOF showed no differences between the samples. This suggests that inactivated PHM was not covalently decorated by an unlabeled fragment from either of these olefins. In other words, upon inactivation, the phenyl ring of [<sup>14</sup>C]-cinnamate or *N*-dansyl-4-aminocinnamate was not lost in some manner yielding modified PHM without incorporation of the <sup>14</sup>C or the dansyl group. Despite the

lack of labeling upon inactivation, our modeling and tiopronin protection studies indicate that the cinnamates are binding at the PHM active site.

The relatively low  $(k_{\text{inact}}/K_{\text{I}})_{\text{obs}}$  values for cinnamate and the cinnamate analogs indicate that these compounds are more likely to dissociate from the PHM–inactivator complex than inactivate PHM (Table 2). A comparison of the inactivation kinetic parameters (Table 2) to the relative C $_{\alpha}$ –H bond dissociation energies (Table 3) illustrates that there is no clear correlation between these values. For example, the C $_{\alpha}$ –H bond dissociation energy of *N*-dansyl-4-aminocinnamate is approximately 2 kcal/mol higher than that of cinnamate, yet  $(k_{\text{inact}}/K_{\text{I}})_{\text{obs}}$  value for *N*-dansyl-4-aminocinnamate is ~500-fold higher than the  $(k_{\text{inact}}/K_{\text{I}})_{\text{obs}}$  value for cinnamate. The major difference in the  $(k_{\text{inact}}/K_{\text{I}})_{\text{obs}}$  ratios between *N*-dansyl-4-aminocinnamate and cinnamate stems from the contribution of binding affinity of each to PHM:  $K_{\text{I,obs}} = 4$  mM for cinnamate and 10  $\mu$ M for *N*-dansyl-4-aminocinnamate. In sum, our data suggest that PHM-mediated inactivation by the cinnamate and the cinnamate analogs does not result from C $_{\alpha}$ –H bond cleavage: (a) no O $_2$  consumption was observed when PHM was incubated with cinnamate in the presence of ascorbate and O $_2$ , (b) no evidence that an oxidized cinnamate is generated by incubation with PHM under turnover conditions, (c) no clear correlation between the inactivation kinetics and the C $_{\alpha}$ –H bond dissociation energies, (d) no kinetic isotope effect for the inactivation of PHM with perdeuterated cinnamate and (e) no labeled protein found when PHM was inactivated with [ $^{14}\text{C}$ ]-cinnamate or *N*-dansyl-4-aminocinnamate. Any mechanism that accounts for the cinnamate-mediated inactivation must be consistent with the data showing that inactive PHM has not been modified in an irreversible fashion, that cinnamate is not a PHM substrate, and that inactivation requires that PHM exist in a catalytically active state.

Like the chemistry proposed for the olefinic inactivation of D $\beta$ M and P $_{450}$ , it has been suggested that olefinic inactivation of PHM results from the formation of a non-epoxide oxidized species, likely an olefin-derived radical<sup>25,33,35–38,64</sup>. This hypothesis is reasonable given that inactivation is turnover-dependent and that the PHM-catalyzed hydroxylation reaction likely proceeds through a substrate-based radical<sup>39–42</sup>. However, this proposed chemistry is inconsistent with our data and is thermodynamically challenging. The formation of a vinyl radical from cinnamate is ~80 kJ/mol higher than formation of an allylic radical from PBA<sup>70</sup>. It is not clear that any of the reduced oxygen species proposed to form during PHM catalysis are of sufficient strength to generate a vinyl radical from cinnamate.

We propose that inactivation of PHM occurs via the Michael addition of an active site nucleophile to cinnamate (Figure 6).  $\alpha,\beta$ -Unsaturated carbonyls are known Michael acceptors<sup>71</sup> and this chemistry is reversible, meaning that the covalently bound cinnamate could have been lost during the procedures used to evaluate inactive PHM. This would account for PHM being unlabeled after inactivation by either [ $^{14}\text{C}$ ]-cinnamate or *N*-dansyl-4-aminocinnamate. The tiopronin protection studies and our modeling results indicate that the cinnamates bind in the active site. Potential active site nucleophiles that could serve as Michael donors include Lys-134, Glu-313, and Tyr-318<sup>36,53,62,72</sup>. Although methionine is a weak Michael donor, the potential reaction between either of the two important active site methionine residues, Met-109 or Met-314<sup>53,62,73</sup>, and the olefin might

explain olefinic inactivation of PHM. A Met-olefin Michael adduct would be readily reversible; thus, accounting for the lack of PHM labeling by [ $^{14}\text{C}$ ]-cinnamate or *N*-dansyl-4-aminocinnamate. Formation of a reversible Michael adduct between an active site nucleophile and cinnamate is consistent with the lack of a kinetic isotope effect on  $k_{\text{inact,obs}}$  because  $\alpha$ -secondary deuterium effects are small ( $\sim 20\%$ , see reference<sup>74</sup>) and the lack of significant substituent effects on  $k_{\text{inact,obs}}$  because electron-donating or electron-withdrawing groups at the para-position of cinnamate would have only a small effect on the electropositive nature of the cinnamoyl  $\beta$ -carbon. The  $\beta$ -carbon of cinnamate is the most electropositive and is the carbon atom most susceptible to nucleophilic attack.

While the reversible Michael addition of an active site nucleophile to cinnamate is one plausible mechanism to explain PHM inactivation, two questions remain. Why does cinnamate inactivation require turnover conditions and why does cinnamate-inactivated PHM remain inactive? Studies of the kinetic mechanism show that the  $\text{O}_2$  and the oxidizable substrate only bind after the reduction of enzyme-bound  $2\text{Cu(II)}$  to  $2\text{Cu(I)}$  by ascorbate<sup>75</sup>. We found that  $\text{O}_2$  binds *after* hippurate or benzaldehyde iminoxy acetate<sup>76</sup> rendering the  $\text{O}_2$ -requirement for cinnamate inactivation perplexing. Either the order of binding for cinnamate is different, requiring  $\text{O}_2$  to bind to reduced PHM before cinnamate can bind, or  $\text{O}_2$  binding to the reduced PHM–cinnamate complex results in a subtle rearrangement in the active site to facilitate cinnamate attack by the active site nucleophile. The lack of reactivation of cinnamate-inactivated PHM would result from a relatively slow off-rate from native PHM. Thus, dialysis or dilution of the cinnamate-inactivated PHM would not show a significant regain in activity, consistent with our data. However, loss of the PHM native structure by denaturation or proteolysis would significantly increase the off-rate for cinnamate accounting for the lack of label in denaturated PHM after inactivation with either  $^{14}\text{C}$ -cinnamate or *N*-dansyl-4-aminocinnamate. Further complicating these experiments is the loss of PHM activity under turnover conditions<sup>77</sup> meaning that reactivation of cinnamate-inactivated PHM under non-denaturing conditions cannot attain 100% of the original activity.

Another potential explanation for PHM inactivation is the cinnamate-dependent removal of copper from the enzyme. Copper-free PHM is catalytically inactive<sup>53,62,78</sup>. Cinnamates are known to form complexes with both  $\text{Cu(I)}$ <sup>79,80</sup> and  $\text{Cu(II)}$ <sup>81</sup> and copper removal/chelation has been attributed to specific PHM<sup>61</sup> and  $\text{D}\beta\text{M}$  inhibitors<sup>68</sup>. PHM inactivation requires turnover; thus, inactivation of PHM by cinnamate could result from the formation of a relatively high affinity cinnamate– $\text{Cu(I)}$  complex. Our data are inconsistent with the simple removal of the copper from PHM to yield inactive apo-enzyme because this mode of inactivation would be reversible<sup>78</sup>. The time-dependence in the cinnamate inactivation could result in the slow accumulation of a tight-binding  $\text{E-2Cu(I)-cinnamate}$  complex. The cinnamate would have to bind to  $\text{PHM-2Cu(I)}$  with sufficient affinity to explain the irreversibility of the inactivation. PHM denaturation would result in release of the cinnamate accounting for the lack of enzyme labeling with either [ $^{14}\text{C}$ ]-cinnamate or *N*-dansyl-4-aminocinnamate. Again, the  $\text{O}_2$ -dependence of the cinnamate-mediated activation is problematic. Either cinnamate only binds to the  $\text{E-2Cu(I)-O}_2$  complex to form an  $\text{E-2Cu(I)-O}_2$ –cinnamate complex or  $\text{O}_2$  binding results in a subtle conformational change that facilitates the formation of the a  $\text{E-2Cu(I)-O}_2$ –cinnamate complex. It is entirely possible

that the cinnamate-mediated inactivation of PHM results from a combination of both the formation of a Michael adduct between an active site nucleophile and cinnamate and the formation of tight enzyme-bound Cu(I)-cinnamate complex. Alternatively, Michael addition of an active site nucleophile could also subtly alter the PHM structure to enable the formation of a tight enzyme-bound Cu(I)-cinnamate complex that decays slowly relative to the retro-Michael reaction.

The Michael addition of an active site nucleophile to an unsaturated carbonyl compound might also account for the inactivation of PHM by the olefinic inactivators. The inactivation by PBA is more complex because, in contrast to the other olefinic inactivators, PBA can serve as a PHM substrate. Yet, PHM is not labeled by PBA<sup>37</sup>. We propose that the PBA-mediated inactivation of PHM occurs by more than one process. Inactivation by PBA could occur via Michael addition/Cu(I) chelation, like the chemistry we have outlined above, and via a reaction between an active site amino acid and PBA-derived radical<sup>35-37</sup>. PHM inactivation by a PBA-derived radical would lead, most likely, to a covalent bond between PBA and the enzyme. Hence, inactivation by [<sup>3</sup>H]-PBA should yield [<sup>3</sup>H]-enzyme, which was not found. Based on this result, we suggest that Michael addition/Cu(I) chelation is the predominant inactivation chemistry and that inactivation via a PBA-derived radical only accounts for a small percentage of the inactivation yielding levels of labeled enzyme that were below the level of detection.

In conclusion, we have demonstrated that cinnamate and other cinnamate analogs are capable of PHM inactivation. The most likely explanation for the inactivation of PHM mediated by the cinnamates is probably the result of the reversible Michael addition of an active site nucleophile at the  $\alpha,\beta$ -unsaturated carbonyl of the cinnamates. The slow accumulation of a tight and inactive PHM-2Cu(I)-cinnamate-O<sub>2</sub> complex could also contribute to the net inactivation of PHM. Similar chemistry may account for the inactivation of PHM by other olefins. The development of a drug possessing an  $\alpha,\beta$ -unsaturated carbonyl should be evaluated as a PHM inactivator, an issue of particular concern given the importance of PHM in  $\alpha$ -amidated peptide hormone biosynthesis. Also, these findings could prove therapeutically relevant as PHM dysfunction is correlated to a number of human diseases<sup>17-22</sup>, meaning that PHM could be a target for the development of novel olefinic-based drugs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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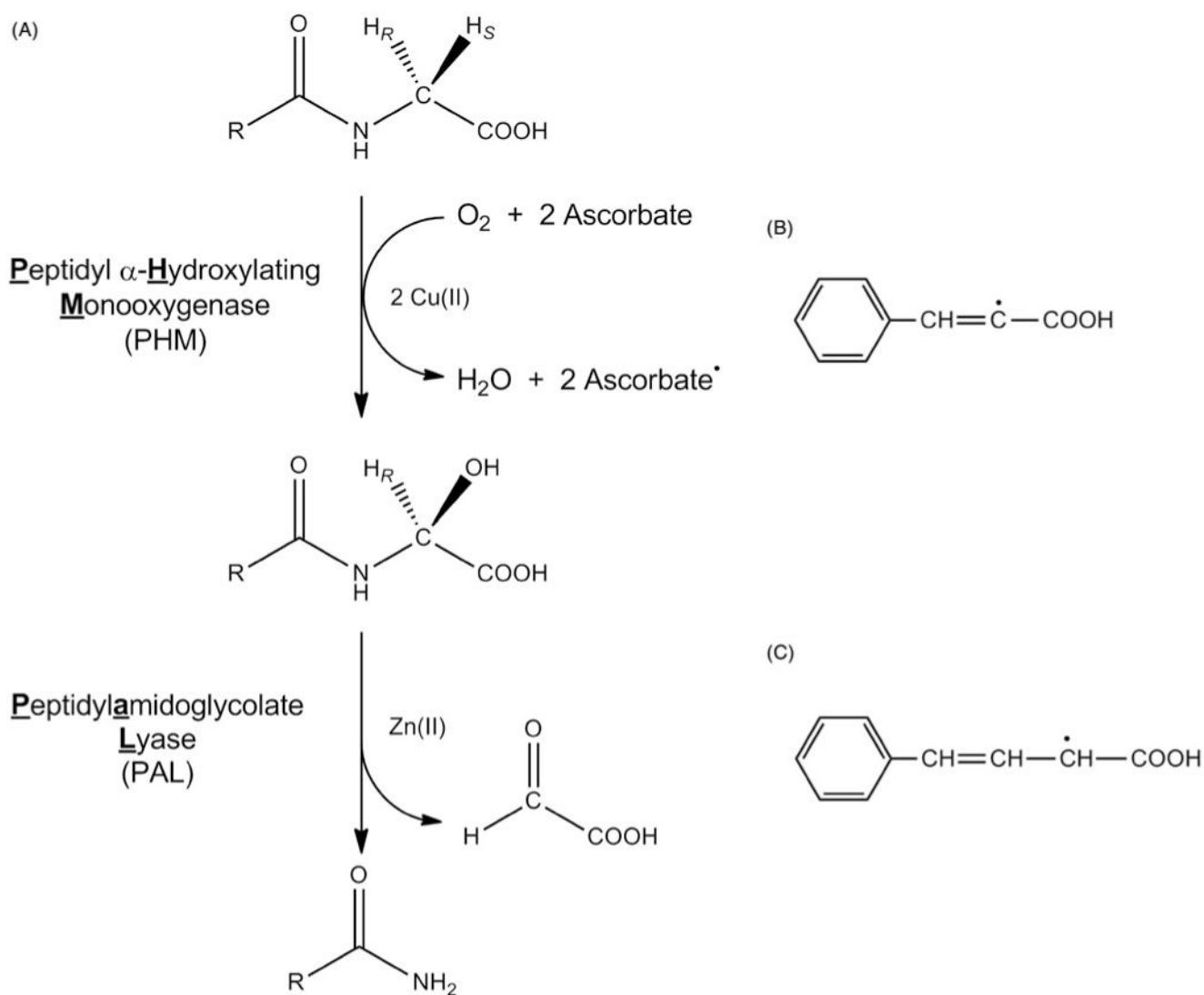
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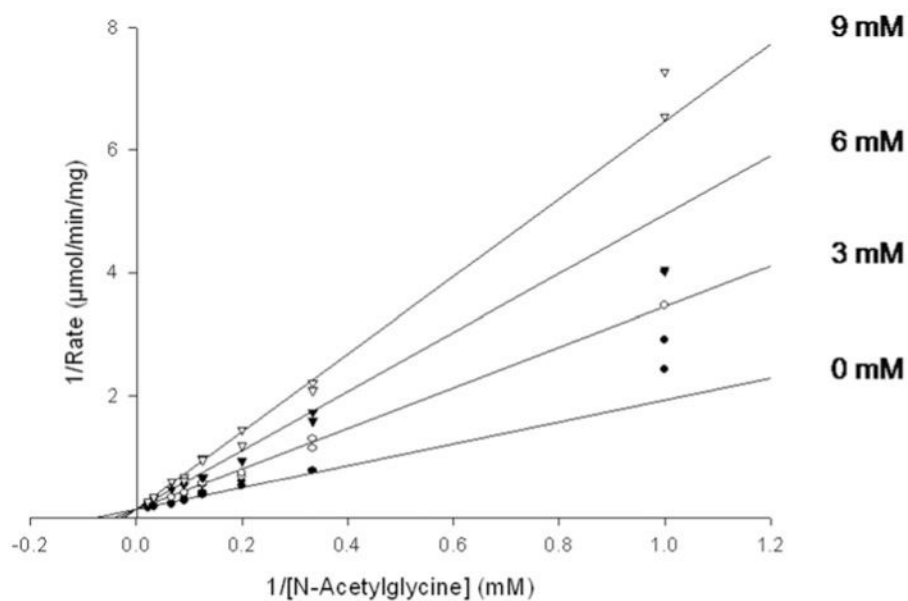
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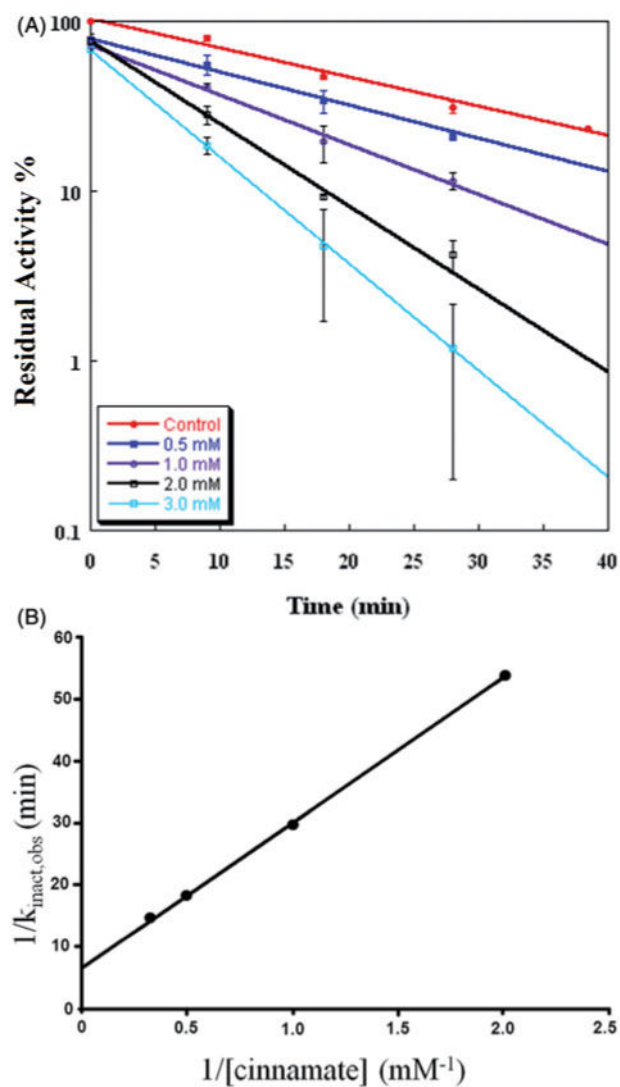
**Figure 1.**

The reactions catalyzed by PHM and PAL (A), the cinnamate-derived vinyl radical (B) and the 4-phenyl-3-buteneoate-derived allylic radical (C). Bifunctional PAM is comprised of separate monofunctional enzymes, PHM and PAL. *In vivo*, the substrate for PHM and PAL is a C-terminal glycine extended peptide ( $R = \text{a peptide}$ ) to generate an  $\alpha$ -amidated peptide.



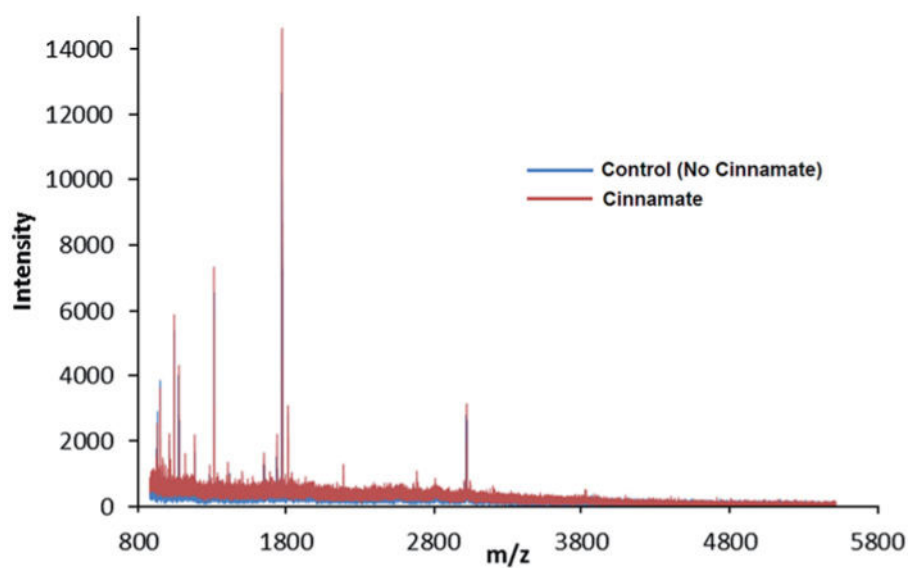
**Figure 2.** Inhibition of PHM by cinnamic acid. Assays were performed as described in “Methods” section for the initial rate determination. The points are initial rates and the lines were computer fit using SigmaPlot 9.0. Initial rates of O<sub>2</sub> consumption were measured for 3 min, during which the inactivation mediated by cinnamate makes a negligible contribution to the observed inhibition.



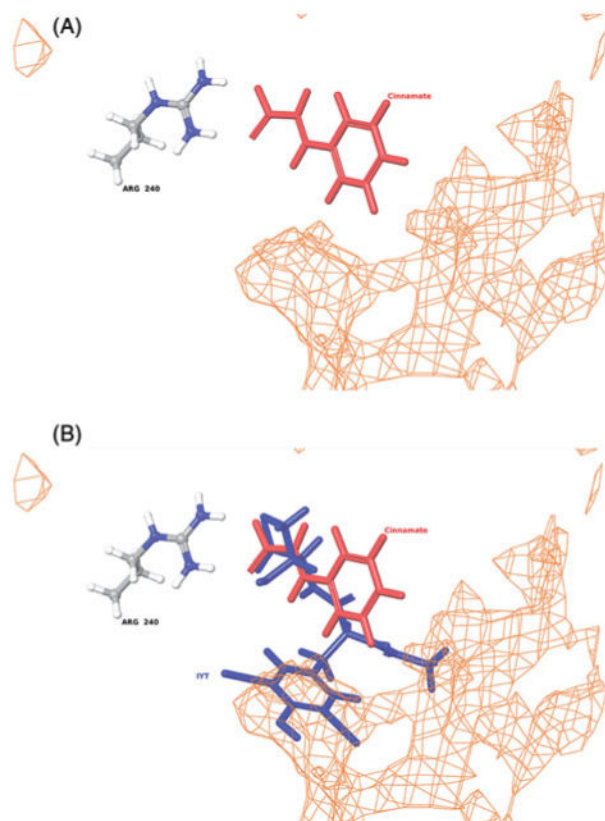


**Figure 3.**

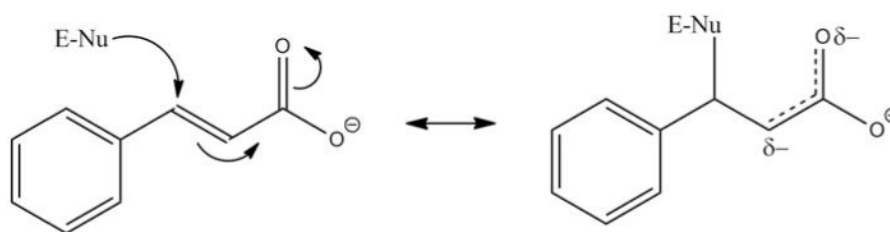
Inactivation of PHM by cinnamic acid. (A) PHM was incubated at 37 °C in the presence of the indicated concentration of cinnamate as described in the “Methods” section. At the indicated times, an aliquot was removed and assayed for residual O<sub>2</sub> consumption activity. Lines are linear regression fits to the equation:  $\log[(v_t/v_0)] = (-k_{\text{obs}}/2.303)t + C$ , where  $v_0$  is the average initial rate of O<sub>2</sub> consumption in the absence of cinnamate,  $v_t$  is the initial O<sub>2</sub> consumption rate at time =  $t$ , and  $C$  is a constant which should be within experimental error of 2.0. The error bars represent the standard deviation of the duplicate measurements. (B) A reciprocal replot of the inactivation data from panel A.



**Figure 4.** MALDI-TOF overlay of trypsin-digested active and cinnamate-inactivated PHM. The catalytically active control is shown in blue, while the inactivated PAM is shown in red. Inactivation was achieved through incubation with cinnamate as described in the “Methods” section. This figure is best viewed in color, which is only available online.

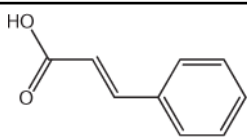
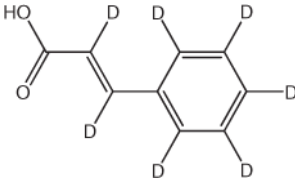
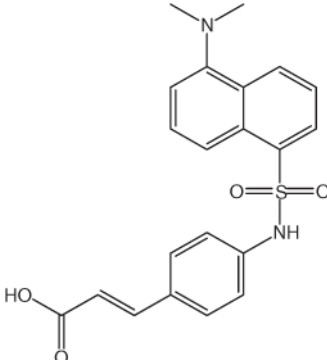
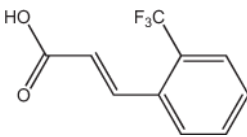
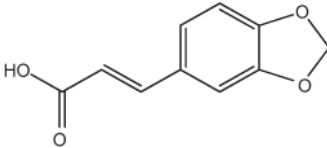
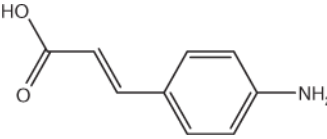
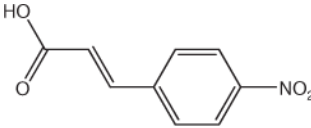


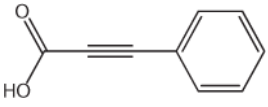
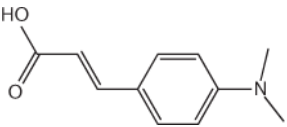
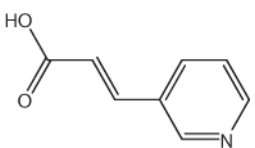
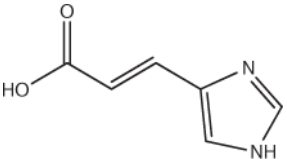
**Figure 5.** Binding of cinnamate into the PHM active site (A) and the relative orientation of cinnamate to bound substrate (*N*-α-acetyl-3,5-diiodotyrosylglycine, IYT) in PHM (B). The orientation of both the IYT (blue) and cinnamate (red) ligands is superimposed to compare recognized electrostatic (Arg-240) and hydrophobic (orange mesh) interactions near the Cu<sub>M</sub> domain associated with productive ligand binding<sup>53,62</sup>. PHM contains two bound copper atoms, Cu<sub>M</sub> and Cu<sub>H</sub><sup>53</sup>.



**Figure 6.** Proposed mechanism for the cinnamate-mediated inactivation of PHM. E-Nu represents an active site nucleophile, possibilities include Lys-134, Glu-313, Tyr-318 Met-109 or Met-314.

**Table 1** $K_{I,obs}$  values for the inhibition of PHM by cinnamate and the cinnamate analogs<sup>a</sup>.

Name	Structure	$K_{I,obs}$ (mM)
Cinnamic acid		$4 \pm 0.2$
Perdeuterated cinnamic acid		$4 \pm 0.3$
<i>N</i> -Dansyl-4-aminocinnamic acid		$0.01 \pm 0.001$
2-Trifluoromethylcinnamic acid		$0.2 \pm 0.02$
3,4-Methylenedioxcinnamic acid		$0.3 \pm 0.04$
4-Aminocinnamic acid		$0.5 \pm 0.06$
4-Nitrocinnamic acid		$0.6 \pm 0.04$

Name	Structure	$K_{I,obs}$ (mM)
Phenylpropionic acid		$2 \pm 0.3$
<i>N,N</i> -Dimethyl-4-aminocinnamic acid		$3 \pm 0.5$
3-(3-Pyridyl)acrylic acid		$6 \pm 0.6$
Urocanic acid		$(1 \pm 0.2) \times 10^1$



**Table 2**Values for  $k_{\text{inact,obs}}$  and  $(k_{\text{inact}}/K_{\text{I}})_{\text{obs}}$  for cinnamate and selected cinnamate analogs.

Compound	$k_{\text{inact,obs}}$ ( $\text{min}^{-1}$ )	$(k_{\text{inact}}/K_{\text{I}})_{\text{obs}}$ ( $\text{mM}^{-1} \text{min}^{-1}$ )
Cinnamic acid	$0.2 \pm 0.02$	$0.04 \pm 0.006$
<i>N,N</i> -Dimethyl-4-aminocinnamic acid	$0.08 \pm 0.01$	$0.03 \pm 0.005$
4-Aminocinnamic acid	$0.03 \pm 0.005$	$0.06 \pm 0.01$
<i>N</i> -Dansyl-4-aminocinnamic acid	$0.2 \pm 0.03$	$(2 \pm 0.3) \times 10^4$
Perdeuterated Cinnamic acid	$0.2 \pm 0.01$	$0.04 \pm 0.004$

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**Table 3**

Relative bond dissociation energies calculated for selected PHM inhibitors or inactivators.

Name	kcal/mol
Cinnamic acid	0
<i>N</i> -Dansyl-4-aminocinnamic acid	2
4-Aminocinnamic acid	9
4-Nitrocinnamic acid	13

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