Steady State and Theoretical Investigations of Peptidylglycine α-Amidating Monooxygenase (PAM)

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Steady State and Theoretical Investigations of Peptidylglycine α-Amidating Monooxygenase (PAM)

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Dedications

I would like to dedicate this to my family, but most importantly my wife and daughter. Without the support and encouragement of my family I would have never made it through these difficult years in graduate school. To my wife, I would like to thank you for your patience, support, and unwavering faith in me. You and our daughter truly drive me to be all that I can. You are my best friend, and I can’t imagine my life without you.
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I would like to thank Dr. David Merkler for giving me the opportunity to work in his laboratory. I am thankful for your guidance and for what you have taught me in both life and science. I appreciate all of the opportunities you have given me. I would also like to thank the Merkler lab members. It’s been an interesting journey, and I don’t know that I could have made it without As the Lab Burns. I wish you all the best of luck. Lastly, I’d like to thank NRM and SEC for the fun times we shared at Savy Jack’s, in lab, and at the gym. I definitely wouldn’t have made it through without you two.
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Steady State and Theoretical investigations of Peptidylyglycine α-Amidating Monooxygenase

Edward W. Lowe, Jr.

ABSTRACT

Approximately 50% of all known peptide hormones are post-translationally modified at their C-terminus. These peptide hormones are responsible for cellular functions critical to survival. Peptidylglycine α-amidating monooxygenase (PAM) is a bi-functional enzyme which catalyzes the conversion of peptide pro-hormones to peptide hormones. PAM is the only known mammalian enzyme that catalyzes the necessary α-amidation to activate these peptide hormones.

PAM has previously been found to perform N-dealkylation, as well as O-dealkylation. We report here that a novel chemistry for PAM, S-dealkylation, has now been shown. PAM was able to catalyzes the hydroxylation and subsequent dealkylation for a series of substituted 2-(phenylthio)acetic acid analogs, leaving a product containing a free thiol capable of coordinating to copper(I).

A series of cinnamic acid derivatives have been investigated as turnover dependent inactivators of PAM. It was shown that the inactivating compounds contained electron donating substituents. All compounds bound competitively versus substrate, though no catalytic activity was noted when tested as substrates. Although no $D_k_{\text{inact}}$ was observed when using perdeuterated cinnamic acid, one cannot rule out hydrogen abstraction from the Ca as this step may not be rate limiting for inactivation. This suggests that the activated oxygen species generated at CuM may be sufficiently reactive to abstract a hydrogen from an alkene to generate a vinyl radical.
Substrate activation is believed to be facilitated by a Cu(II)-superoxo complex formed at CuM. Hydrogen abstraction from the Cα is hypothesized to generate a radical, though this has never been demonstrated spectrometrically. We report here further evidence for the generation of a Cα radical by comparing log($V_{\text{max}}/K_{O2}$) vs $\sigma^+$ for a series of ring-substituted 4-phenyl-3-butenolic acids.

Lastly, a computational study was carried out to probe for a possible binding pocket for the reductant, ascorbate. Though crystal structures have argued that reduction of the enzyme-bound coppers is collisional, kinetic data for inhibitors competitive against ascorbate indicates that a discrete binding pocket may exist. Our study suggests a specific site for binding and provides free energy calculations in agreement with experimental values for binding constants.
Peptide Hormones

Approximately 50% of all known mammalian peptide hormones are α-amidated. In insects, over 90% of peptide hormones are α-amidated. The amino acid prohormone sequence of these peptides contains a C-terminal glycine. The amidation of the carboxy-terminus is essential for activation of these peptide hormones as the glycine-extended precursors are generally >1000-fold less potent than the α-amidated peptides [1]. Some of these amidated hormones act as neurotransmitters. Many α-amidated peptide hormones have been found to be over-expressed in human disease states such as substance P in rheumatoid arthritis [2, 3], luteinizing hormone-releasing hormone and vasoactive intestinal peptide in cancer[4, 5], and corticotropin-releasing factor in anxiety and depression[6]. The fact that the precursor sequences for these essential amidated hormones all contained a C-terminal glycine suggested that the C-terminal amide biosynthesis required the action of a specific enzyme to activate these peptide prohormones through the functionalization of the glycine (Figure 1.1). In 1982, the enzyme responsible for the conversion of glycine-extended peptides to the corresponding peptide-amide was discovered in porcine pituitary[7].

PAM: The α-Amidating Enzyme

Peptidylglycine α-amidating monooxygenase (PAM) is a bifunctional copper-monooxygenase. PAM is found within neuronal and endocrine cells, neurosecretory vesicles, and the highest concentrations are found in the atrium of the heart [8-11]. PAM is primarily responsible in vivo for the activation of peptide hormones through the conversion of the glycine-extension to the corresponding amide. PAM also plays a role in the biosynthesis of fatty acid amides [12-15]. PAM is the only mammalian enzyme known to catalyze this amidation reaction.
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PAM (E.C. 1.14.17.3) consists of two hetero-domains which are peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidylglycine amidoglycolate lyase (PAL) (Figure 1.2). The PHM domain has been deeply studied primarily due to its similarity in both reaction and structure to another copper-dependent monooxygenase, dopamine β-monooxygenase [16, 17]. Dopamine β-monooxygenase (DβM, E.C. 1.14.17.1) catalyzes the stereospecific conversion of dopamine to norepinephrine (Figure 1.3) [16, 18]. This tetrameric protein exists in both membrane and soluble forms within neurosecretory vesicles of the sympathetic nervous system [19]. While dopamine is a neurotransmitter in the central nervous system, its functionalization to norepinephrine by DβM allows for its functioning in the sympathetic nervous system which consists of neuronal axons that interact with smooth muscle and the catecholamine secreting cells within the adrenal medullae [20].

**PHM and DβM**

PHM and DβM are structurally similar. The enzymes share a 28% identity and 40% sequence similarity in the catalytic core [17, 21]. Both contain a large, solvent accessible active site flanked by two non-coupled copper atoms [22-24]. PHM catalyzes the copper-, O₂-, and ascorbate-dependent hydroxylation of a glycyl Cα while DβM catalyzes the copper-, O₂-, and ascorbate-dependent hydroxylation of a benzylic carbon (Figure 1.4) [16, 22, 25-27]. While PHM has been successfully crystallized, DβM has not [28]. The crystal structure of PHM reveals it to be a prolate ellipsoid composed of two 9-stranded β-sandwich domains (Figure 1.5). The domains are approximately of equivalent size and are held together by a 500 Å² interface. The active site is a large, solvent accessible cleft residing at the interdomain interface with two bound coppers, one on each side of the cleft (Figure 1.6). Each domain is centered around their respective copper atom. Each of the two coppers has a different ligand set. One copper center,
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CuM, has two histidine \( N_\epsilon \) ligands and a methionine sulfur ligand (H242, H244, M314). The remaining copper center, CuH, has three histidine \( N_\delta \) ligands (H107, H108, H172) (Figure 1.7). This is also the case in D\( \beta \)M as one copper is coordinated to three histidines and the other to two histidines and a methionine. The enzyme was co-crystallized with \( N \)-acetyl-3,5-diiodotyrosyl-threonine, a poor substrate for PHM. The substrate appears to form a salt bridge between the carboxy-terminus and the guanidino group of arginine 240 (Figure 1.8). This places the substrate in close proximity to the CuM where \( O_2 \) has been found to bind and where activation of \( O_2 \) is thought to occur. Hydrogen bonding between the glycyl amide-hydrogen and the side chain oxygen of N316 also plays a role in the proper positioning of the substrate. Crystal structures for oxidized, reduced, and pre-catalytic forms of PHM with substrate bound have been solved [28-30]. The crystal structures are surprisingly identical with no differences seen between the oxidized and reduced form and an rmsd value of 0.27 (Figure 1.9). This suggests that movement or closure of the active site during catalysis does not occur. These structures also revealed that the copper atoms are \( \sim 10.6 \) Å apart which is in good agreement with previous extended X-ray absorption fine structure (EXAFS) data for D\( \beta \)M which suggested the copper atoms were more than 4 Å apart [21, 28, 30-32]. These data along with electron paramagnetic resonance (EPR) data suggests that PHM and D\( \beta \)M are non-coupled di-copper enzymes or ‘non-blue’/type-II enzymes [22, 33, 34]. The geometries of the two copper sites were found to change upon reduction. CuM is observed to have a square pyramidal geometry while in the oxidized form while CuH is square planar. Upon reduction, CuM becomes tetrahedral and CuH becomes T-shaped. The bond length of CuM-SM314 has been shown to tighten in the reduced form compared to oxidized enzyme according to EXAFS data suggesting that the oxidized form is more distorted
tetrahedral than square planar [34]. EXAFS data also suggests that CuH is coordinated to only two of the three histidine residues in the reduced form.

Both PHM and DβM catalyze very similar reactions. The PHM reaction involves hydrogen abstraction from a substrate by some activated Cu-O species followed by the subsequent stereospecific hydroxylation of the pro-S glycyl Ca. The DBM reaction also involves the abstraction of hydrogen, but does so from a benzylic carbon followed by pro-R hydroxylation. In both cases, the mechanism is believed to involve electron transfer from CuH to CuM.

**PHM/DβM Postulated Mechanisms**

The first step involved for catalysis is the irreversible reduction of both enzyme bound coppers by ascorbate allowing di-oxygen and substrate to bind to the reduced enzyme [16, 21, 35, 36]. This reduction is a “ping-pong” mechanism. Burst phase kinetic experiments with DβM have shown that pre-reduced enzyme is capable of hydroxylating substrate with the amplitude of the pre-steady state burst being equivalent to the concentration of enzyme [37-39]. This indicates that the chemical step is much faster than product release and also allowed for re-oxidation with product release to be observed. A temperature dependence on primary and secondary intrinsic isotope effects for Ca-H cleavage has also been observed for PAM. This non-classical behavior suggests that quantum tunneling is involved in hydrogen transfer [40].

$^{18}$O kinetic isotope effects have been used to probe the nature of the activated Cu/O species responsible for hydrogen abstraction. These studies on both PHM and DβM only provide information up to and including the O-O cleavage step due to the quantum mechanical nature of hydrogen transfer [41]. When coupled with substrate deuterium isotope effects, the
data for PHM and DβM indicates that C-H bond cleavage occurs prior to that of O-O necessary for hydroxylation [42-45]. This suggests that a Cu(II)-hydroperoxo is being formed during hydrogen abstraction but lends no evidence as to what the hydroxylating species may be. Rapid freeze quench EPR data also suggests that a copper-superoxo species is formed, as O-O and C-H cleavage are tightly coupled [46]. The data suggest that the substrate intermediate resulting from Cu(II)-superoxo abstraction of hydrogen would be a radical intermediate. Evidence for a radical mechanism has been found in DβM [47-49]. This evidence has been applied towards the PHM mechanism though it has not been proven directly for PHM.

Several competing reaction mechanisms have been proposed for PHM and DβM. Two of these competing mechanisms both support a copper-superoxo nucleophile for hydrogen abstraction. While the nature of the hydrogen acceptor is in agreement, the initial geometry of the di-oxygen species to copper is in question. Spectroscopic data has indicated a side-on/η² species but was based on model studies and not PHM or DβM [50, 51]. When compared to the end-on/η¹ species, the side-on Cu(II)-superoxo was determined computationally to be more thermodynamically favorable [52, 53]. However, crystallographic evidence of a pre-catalytic state of PHM with good resolution (1.85 Å) has found the end-on/η¹ species present (Figure 1.10) [28]. Upon forming either disputed Cu-superoxo species, both mechanisms proceed to abstract hydrogen to form a Cu(II)-hydroperoxo species. This hydroperoxo species is coordinated to copper in an end-on/η¹ geometry. The competing mechanisms again diverge as the side-on/η² mechanism predicts direct hydroxylation of the resulting substrate radical followed by radical recombination. The net effect is reduction of the copper(II)-hydroperoxo species and release of the hydroxylated product. Electron transfer would then occur from CuII to complete the reaction.
On the other hand, the end-on/η¹ mechanism postulates that the Cu(II)-hydroperoxo species is reduced by an electron transfer event prior to hydroxylation of the substrate radical. This electron provided by the Cu_H site allows for homolysis of the Cu(II)-O-OH yielding a Cu(II)—O• species. This newly formed Cu/O• would then recombine with the substrate radical intermediate resulting in a covalent, inner-sphere alcohol intermediate. Product release then occurs via hydrolysis of this intermediate. The proposed mechanisms are compared in Figure 1.11.

While experimentalists agree that the hydrogen abstracting species is, indeed, a copper-superoxo, several theoreticians have proposed that a different activated Cu/O species is responsible for this chemistry. Using a series of molecular dynamics (MD) and mixed quantum mechanics molecular mechanics (QM/MM) calculations, two separate mechanisms have been proposed. The species proposed as responsible for hydrogen abstraction is a reduced copper-superoxo. This suggests that di-oxygen reduction and substrate activation are uncoupled which is in direct contrast to experimental data. QM/MM simulations suggest that Cu(III)-oxide/Cu(II)-oxyl species are thermodynamically favored over the superoxo/hydroperoxo species proposed by experimentalists [54-56]. These theoretical mechanisms suggest that electron transfer precedes hydrogen abstraction allowing Cu(II)-superoxo to acquire two protons from solvent. Water is then released from the Cu(II)-O-OH₂ species leaving either a Cu(II)-oxyl quartet or a Cu(II)-oxyl triplet species to abstract hydrogen from the substrate. The quartet is proposed to concertedly abstract the substrate hydrogen and hydroxylate the radical intermediate with a spin inversion to the doublet ground state occurring upon substrate oxidation[54]. This suggests that substrate oxidation and product release happen simultaneously. A water molecule would then bind to the remaining Cu(II) to restore proper geometry in the oxidized state. The triplet Cu(II)-oxyl is
believed to undergo a spin inversion event to the singlet ground state upon substrate C-H oxidation driving concerted product release [55, 56]. These two theoretical mechanisms are compared in Figure 1.12.

**Peptidylglycine Amidoglycolate Lyase**

The lyase domain in PAM responsible for carbinolamide dealkylation of the α-hydroxyglycine PHM product resulting in the corresponding amide and glyoxylate is the PAL domain. This 33 kDa monomer is zinc-, calcium-, and iron-dependent and bound to the C-terminus of the PHM domain. Although the complete PAL mechanism is unknown, it is believed to proceed through a zinc-hydrolase-type reaction [57]. The iron is involved in a tyrosine bridged Zn(II)-Fe(III) complex. Mutation of this tyrosine (Y564) results in PAL inactivation and the inability to bind iron. No substrate channeling between PHM and PAL has been observed. While the inclusion of the PAL domain in PAM provides the key difference between PAM and DβM, DβM has also been noted to catalyze several N-dealkylation reactions solely through hydroxylation like that of benzylic N-substituted analogues [58]. PAM and DβM have also both demonstrated sulfoxidation while PAM has demonstrated O-dealkylation [59].

**Introduction to Chapters**

PAM has previously been found to perform N-dealkylation, as well as O-dealkylation. We report here that a novel chemistry for PAM, S-dealkylation, has now been shown. PAM was able to catalyzes the hydroxylation and subsequent dealkylation for a series of substituted 2-(phenylthio)acetic acid analogs, leaving a product containing a free thiol capable of coordinating to copper(I).
A series of cinnamic acid derivatives have been investigated as turnover dependent inactivators of PAM. It was shown that the inactivating compounds contained electron donating substituents. All compounds bound competitively versus substrate, though no catalytic activity was noted when tested as substrates. Although no $D_{k_{\text{inact}}}$ was observed when using perdeuterated cinnamic acid, one cannot rule out hydrogen abstraction from the Cα as this step may not be rate limiting for inactivation. This suggests that the activated oxygen species generated at CuM may be sufficiently reactive to abstract a hydrogen from an alkene to generate a vinyl radical.

Substrate activation is believed to be facilitated by a Cu(II)-superoxo complex formed at CuM. Hydrogen abstraction from the Cα is hypothesized to generate a radical, though this has never been demonstrated spectrometrically. We report here further evidence for the generation of a Cα radical by comparing $\log(V_{\text{max}}/K_{O2})$ vs $\sigma^+$ for a series of ring-substituted 4-phenyl-3-butenoic acids.

Lastly, a computational study was carried out to probe for a possible binding pocket for the reductant, ascorbate. Though crystal structures have argued that reduction of the enzyme-bound coppers is collisional, kinetic data for inhibitors competitive against ascorbate indicates that a discrete binding pocket may exist. Our study suggests a specific site for binding and provides free energy calculations in agreement with experimental values for binding constants.
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Figure 1.12. The two mechanisms proposed by theoreticians are compared with the quartet to doublet spin inversion above and the triplet to singlet spin inversion below.
Introduction

Peptidylglycine α-amidating monooxygenase (PAM) catalyzes the oxidative cleavage of C-terminal glycine-extended peptide hormones[1], fatty acid precursors [2-5], and bile acids [6] to the corresponding amides and glyoxylate. This two-step amidation proceeds via a copper-, O\textsubscript{2}-, and ascorbate-dependent glycyl Cα hydroxylation followed by a zinc-, iron-, and calcium-dependent dealkylation of the carbinolamide intermediate (Figure 2.1). This bifunctional nature is attributable to the peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidylglycine amidoglycolate lyase (PAL) domains, respectively [7, 8]. This α-amide moiety is a requisite for most active peptide hormones, with the amidated derivatives generally displaying >1000-fold more potency than the glycine-extended precursor[1]. PAM is responsible for the activation of many important neuropeptides such as substance P and oxytocin [9-11]. While this is the primary \textit{in vivo} function of PAM, alternate catalytic functions have been reported including sulfoxidation, and O-dealkylation [12].

PAM/PHM is a potential therapeutic target because of the roles played by α-amidated peptides in disease states. Some examples of α-amidated peptides implicated in disease are luteinizing hormone-releasing hormone (cancer), vasoactive intestinal peptide (cancer), substance P (rheumatoid arthritis), and corticotropin-releasing factor (anxiety and depression). Because prevention of amidation often reduces the efficacy of peptide hormones by ~3 orders of magnitude, broad inhibition of PAM may be overly toxic by blocking the conversion of essential mammalian α-amidated peptides.

Toxicity may, however, prove useful in the possible development of insecticides. Insects possess separate PHM and PAL monomers and lack the bi-functional PAM enzyme [13, 14].
Elimination of expression in *Drosophila melanogaster* has been demonstrated to be lethal [14]. Because the mammalian PHM sequence and *Drosophila* PHM sequence share 41% identity and 52% similarity, sufficient differences may exist to target only the insect enzyme.

Several (2-phenylthio)acetic acid (PTAA) derivatives were investigated as inhibitors of PAM activity. PTAA displays characteristics of a turnover-dependent inactivator of PAM. We now report that PAM can catalyze S-dealkylation. This S-dealkylation leaves a free thiol capable of *in situ* coordination of the active site Cu$_M$ leading to reversible inactivation of PAM. This discovery may give a new direction for the design of PAM inhibitors. The S-Cα moiety allows for the design of PAM specific pro-drugs that rely on PAM turnover for drug activity.

**MATERIALS AND METHODS**

*Materials.*

[(1-H-indole-2-ylcarbonyl)thiol]acetic acid and 2-(phenylthio)acetic acid were purchased from Sigma, 2-(4-chlorophenylthio)acetic acid was purchased from Pfaltz & Bauer, Inc., 2-(4-methylphenylthio)acetic acid was purchased from Aldrich Chem. Co., 2-(benzoylthio)acetic acid, 2-(2-nitrophenylthio)acetic acid, N-benzoyl-D-alanylthioglycolic acid were purchased from Eburon Organics N.V., and bovine catalase was from Worthington. Recombinant rat PAM was a gift from Unigene Laboratories, Inc. All other reagents were of the highest quality from commercial suppliers.
Chapter Two: Novel Chemistry in PAM: S-dealkylation

Synthesis of α-di-deutero-2-(phenylthio)acetic acid

Thiophenol (6.1 mL, 60 mmol) in aqueous sodium hydroxide (20 mL 6M) was reacted with (α-D,D) bromoacetic acid (12.5 gm, 90 mmol) for 2 hr at 50 °C. The reaction was then acidified with dilute HCl(aq), precipitate collected, and re-crystallized twice in hot ethanol.

Determination of $K_{M,\text{app}}$ and $V_{MAX,\text{app}}$ values for 2-(phenylthio)acetic acid (α-H,H and α-D,D), 2-(4-methylphenylthio)acetic acid, 2-(4-chlorophenylthio)acetic acid, and 1-H-indole-2-ylcarbonylthiolacetic acid.

Reactions at 37.0 ± 0.1 °C were initiated by the addition of PAM (20-50 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$_3$)$_2$, 5.0 mM sodium ascorbate, and 0.1 – 6.0 mM of the oxidizable substrate. Initial rates were measured by following the PAM-dependent consumption of O$_2$ using a Yellow Springs Instrument Model 5300 oxygen monitor interfaced with a personal computer using a Dataq Instruments analogue/digital converter (model DI-158UP). $V_{MAX,\text{app}}$ values were normalized to controls performed at 11.0 mM N-acetylglycine.

Inhibition of O$_2$ consumption from N-acetylglycine by 2-(phenylthio)acetic acid Reactions at 37.0 ± 0.1 °C were initiated by the addition of PAM (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$_3$)$_2$, 5.0 mM sodium ascorbate, 8 mM N-acetylglycine, and 0-5 mM 2-(phenylthio)acetic acid. Initial rates were measured by following the PAM-dependent consumption of O$_2$ using a Yellow Springs Instrument Model 5300 oxygen monitor.
Glyoxylate production from 2-(phenylthio)acetic acid derivative substrates

To first determine if the PHM-hydroxylated 2-(phenylthio)acetic acid was a substrate for PAL, 20 µg of PAM was added to a 2.0 mL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 µg/mL catalase, 1.0 µM Cu(NO₃)₂, 5.0 mM sodium ascorbate, and 10 mM 2-(phenylthio)acetic acid. After incubation for 2 hr. at 37°C, the reaction was terminated by the addition 500 µL of 6% (v/v) TFA and duplicate 500 µL aliquots were removed for glyoxylate analysis.

Initial rates of glyoxylate formation from 2-(phenylthio)acetic acid were determined by adding 20 µg of PAM to a 3.0 mL solution containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 µg/mL catalase, 1.0 µM Cu(NO₃)₂, 5 mM sodium ascorbate, and 10 mM 2-(phenylthio)acetic acid. At 30 min. intervals for 150 min., a 500 µL aliquot was removed, added to a vial containing 100 µL of 6% (v/v) TFA to terminate the reaction, and the concentration of glyoxylate formed measured in the acidified samples.

Initial rates of glyoxylate formation from α-hydroxyhippurate, a known PAL substrate, as a function of the 2-(phenylthio)acetic acid concentration were determined by adding 20 µg of PAM to a 1.5 mL solution containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10.0 mM α-hydroxyhippurate, and 0-10.0 mM 2-(phenylthio)acetic acid. 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) TFA to terminate the reaction, and the concentration of glyoxylate formed measured in the acidified samples.

Glyoxylate was determined by the spectrophotometric method of Christman et al. [15] as modified by Katopodis and May [12]. Standard curves of [glyoxylate] vs. A₅₂₀ were constructed in the appropriate buffers using a glyoxylate solution that had been standardized by measuring
the glyoxylate-dependent oxidation of NADH (\(\Delta \varepsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\)) as catalyzed by lactate dehydrogenase.

**RP-HPLC separation of 2-(phenylthio)acetic acid and thiophenol.**

HPLC analyses were performed using a Hewlett-Packard 1100 liquid chromatography system equipped with an auto-sampler and quaternary pump system. Aliquots (10 µL) of the reaction mixture were analyzed using a Phenomenex Luna 5µ C18 (250 x 4.60 mm) column. The mobile phase was a linear 70-100% gradient of 50 mM sodium acetate pH 6/acetonitrile (85/15) in acetonitrile. Analytes were detected at 254 nm.

**Analysis of steady-state kinetic data**

Steady-state kinetic parameters were obtained by Kaleidagraph fit of the initial velocity (v) vs. substrate concentration, ([S]), data to

\[
v = \frac{V_{\text{MAX,app}}[S]}{K_{M,\text{app}} + [S]} \tag{1}
\]

Where \(K_{M,\text{app}}\) is the apparent Michaelis constant for the oxidizable substrate at fixed [ascorbate] and \([O_2]\) concentrations and \(V_{\text{MAX,app}}\) is the apparent maximum initial velocity at saturating [S].

Initial rate studies that resulted in competitive inhibition were also analyzed by Kaleidagraph fit of v vs. [S] as a function of inhibitor concentration to equation 2.

\[
v = \frac{(V_{\text{MAX,app}} [S])}{(K_{M,\text{app}} \left(1 + \frac{[I]}{K_{i,s}} + [S]\right))} \tag{2}
\]
In silico docking

The crystal structure for reduced peptidylglycine α-hydroxylating monooxygenase (PHM) was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/, 1SDW) [16]. 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 hydrogens were added using Maestro (www.schrodinger.com). Further receptor refinements were carried out utilizing ProteinPrep from within Maestro. Glide and Q-site from the FirstDiscovery 3.0 suite (www.schrodinger.com) were used for quantum polarized ligand docking (qpld) to generate highly accurate ligand binding modes [17, 18].

Computational Chemistry

The top pose from the in silico docking studies of thiophenolate was used as input for the geometry optimization of the L₃Cu-thiophenolate system. The Cu₅ ligand set, H242, H244, and M314, were truncated to methyl-capped side chains to lessen the computational expense of geometry optimizing the system. Three systems were thus created. System 1 included the previously mentioned methyl-capped side chains, Cu⁺, and a single water molecule coordinated to the copper (Figure 2.2). System 2 included, once again, the L₃Cu⁺ system, but the coordinated water was replaced by the qpld docked thiophenolate (Figure 2.3). The final system, System 3, contained only the three-residue Cu₅ coordination site along with Cu⁺ (Figure 2.4). The geometries of these two systems were optimized using density functional theory with B3LYP hybrid exchange-correlation and the LanL2DZ+ECP basis set [19-21].
RESULTS AND DISCUSSION

2-(phenylthio)acetic acid as a PAM substrate

The addition of PAM to solutions containing p-Cl-PTAA resulted in the consumption of O₂ (Figure 2.5). The dependence of the initial rate of O₂ consumption on the initial concentration of PTAA, at a fixed concentration of reductant and O₂, is described by equation 1 yielding the steady-state kinetic values for $K_{M,\text{app}}$, $V_{\text{MAX,app}}$, and $(V/K)_{\text{app}}$ included in Table 2.1. Investigation of $\alpha$-d,d-PTAA gave a $^D(V/K)_{\text{app}}$ isotope effect of 1.9 giving further proof that the Cα-H bond cleavage was occurring. Incubation of 10 µg/mL PAM with 10 mM PTAA for 1 hr at 37 °C yielded 96 µM glyoxylate verifying that PTAA is indeed a substrate and alluding to the inactivating species, thiophenol (Figure 2.6).

2-(phenylthio)acetic acid derivatives as PAM substrates and inhibitors

The pattern of O₂ consumption observed for all PTAA-related substrates appeared characteristic of that of a mechanism-based inhibitor indicating turnover followed by enzyme inactivation. Because this inactivation was slow, analyses of initial rates in obtaining the steady-state kinetic parameters is valid. Glyoxylate was also produced in all instances. Those compounds in which glyoxylate was not produced, however, neither consumed O₂ nor inactivated PAM. The derivatives which did not consume O₂ when tested as substrates did inhibit O₂ consumption from N-acetylglycine (Figure 2.7) yielding the steady-state kinetic values as described by equation 2 (Table 2.2). LC/MS of trypsin-digested PAM after incubation with PTAA showed no labeling (data not shown) suggesting that the enzyme was not being covalently modified as is typical of a mechanism-based inhibitor. LC/MS analysis did, however, indicate
Chapter Two: Novel Chemistry in PAM: S-dealkylation

that thiophenol was being produced. An HPLC separation was then developed and thiophenol was successfully found in the reaction mixture (Figure 2.8)

*Product-mediated reversible inactivation*

Thiophenol is a known Cu$^{1+}$ chelator and interacts more weakly with Cu$^{2+}$. It was hypothesized that the thiophenol product either coordinate to active site copper, forming an E-2Cu(II)/(I)-inhibitor complex, or removes the active site copper, forming a copper-free-E and separate Cu(II)/(I)-inhibitor complexes. The copper free form of PAM is inactive [22, 23]. This type of thiol-mediated inhibition is well-documented in both PAM and related enzyme dopamine β-monooxygenase (DβM), both copper-dependent monooxygenases [24-27]. The inhibition of PAM by homocysteine-extended peptides [28] and by captopril [29] has been attributed to sulfur-copper interactions. The inhibition of DβM by captopril, and glutathione has been attributed to *in situ* chelation of the enzyme-bound coppers [30, 31]. If *in situ* chelation were occurring, out-competing the S-Cu interaction leaving the enzyme-bound copper free to once again support catalysis should be possible. Thus, a reaction was initiated with 20 µg of PAM under the previously mentioned condition for monitoring O$_2$ consumption and 5 mM 2-(4-chlorophenylthio)acetic acid. Upon observing the inactivation of PAM, with the rate of O$_2$ consumption matching that of the background prior to initiating the reaction with enzyme, the reaction was spiked with HgCl$_2$ to a final Hg$^{2+}$ concentration of 5 µM. This caused a resumption of O$_2$ consumption of 2 µM, leading to the supposition that the stronger Hg-S interaction was relieving the enzyme-bound copper of the proposed chelating species, thiophenol (Figure 2.9).
Modeling of PTAA, analogs, and thiophenol in the PHM active site

The X-ray structures of oxidized and reduced PHM, the subunit responsible for catalyzing the copper-dependent glycy1 Cα hydroxylation, reveal that the enzyme-bound coppers are \( \sim 11\text{Å} \) apart flanking a solvent-accessible active site cleft [16, 24]. Cu\(_H\) and Cu\(_M\), named for their respective ligands of three histidines (Cu\(_H\)), and two histidines and a methionine (Cu\(_M\)), have different roles in catalysis as Cu\(_H\) is involved in electron transfer while Cu\(_M\) is the site of O\(_2\) activation and substrate hydroxylation. Utilizing the crystal structure of reduced PHM in a pre-catalytic state with O\(_2\) coordinated to Cu\(_M\) and a substrate analog in the active site (Figure 2.10), we were afforded an excellent opportunity to model the sulfur-containing compounds and the proposed product responsible for \textit{in situ} chelation, thiophenol.

The reduced PHM crystal structure, 1SDW [16], was chosen for the docking studies. All ligands were docked along with the proposed thiophenol product and the thiophenolate species (C6H5S\(^-\)), L and L\(^-\) respectively. This thiolate species should account for \( \sim 20\% \) of the product under reaction conditions, pH 6.0. All of the analogs bound similarly to the reduced PHM crystal structure, displaying a salt bridge of the carboxy terminus to the guanidine group of R240 similar to that of the natural glycine-extended substrates of PHM [24]. The greatest dissimilarity in binding modes with that of the biological substrate is the absence of the glycy1 amide hydrogen bonding to the oxygen of N316 [24]. This interaction plays a large role in proper positioning of the Cα-H for H abstraction (Figure 2.11).

\textit{Coordination of Cu\(_M\) by thiophenol}

The binding mode from the qpld docking of thiophenolate was used as the starting structure for the geometry optimization as indicated in the Materials and Methods section. The
optimized geometries for the coordination of Cu\(^{1+}\) to the three residue coordination site in the Cu\(_{\text{M}}\) domain were compared between the L\(_3\)Cu\(^{1+}\)-H\(_2\)O system, the L\(_3\)Cu\(^{1+}\) system, and the L\(_3\)Cu\(^{1+}\)-thiophenolate system to investigate coordination stability and to determine whether the coordination of the thiophenolate would disrupt the L\(_3\)Cu\(^{1+}\) stability. The L\(_3\)Cu\(^{1+}\) uncoordinated system is as expected with coordination distances of 2.01, 1.98, and 2.43 Å to N\(_{\text{H}242}\), N\(_{\text{H}244}\), and S\(_{\text{M}314}\) respectively (Figure 2.12). The L\(_3\)Cu\(^{1+}\)-H\(_2\)O geometry optimized system displaced a tetrahedral geometry with coordination distances of 2.05, 2.01, 2.52, and 2.19 Å for N\(_{\text{H}242}\), N\(_{\text{H}244}\), S\(_{\text{M}314}\), and H\(_2\)O respectively (Figure 2.13). This geometry is in good agreement with experimental values for this coordination state in the Cu\(_{\text{M}}\) domain. The geometry optimization for the final system revealed something unexpected. The thiophenolate anion disrupted the geometry of the Cu\(_{\text{M}}\) and changed the coordination number of the system. The geometry shifts from the typical slightly distorted tetrahedral from the water coordinated system to a trigonal pyramidal 3-coordinate system with the S\(_{\text{M}314}\) 4.75 Å away from Cu\(_{\text{M}}\) (Figure 2.14). The other coordination distances are 2.13, 2.08, and 2.38 for N\(_{\text{H}242}\), N\(_{\text{H}244}\), and S-thiophenolate respectively. This change in coordination number and geometry would greatly reduce the chance of oxygen activation.

**Conclusion**

In conclusion, we have shown that PAM can catalyze the dealkylation of the R-S-C\(_{\alpha}\) moiety leaving a free thiol/thiolate capable of \textit{in situ} coordination to enzyme-bound copper. Recent work by the Merkler group has demonstrated that the inclusion of a sulfur atom in small molecule inhibitors of PAM increases binding affinity regardless of oxidation state \textit{(in press)}. The present work suggests that a pro-drug could be developed allowing PAM to catalyze the
production of the free thiol thus greatly reducing the possibility for unintentional free thiol interactions with other metallo-enzyme prior to contact with PAM.
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Figure 2.4. The CuM domain with the L₃ side chains methyl-capped and coordinated to Cu⁺. Note, the carbons are displayed in teal, the nitrogens in blue, the hydrogens in white, the oxygen in red, the sulfur in yellow, and the copper in orange.
Figure 2.5. The PAM-Dependent Consumption of O₂ in the Presence of 2-(4-chlorophenylthio)acetic acid. O₂ consumption at 37°C was initiated by the addition of 20 μg of PAM (A) to a 2.0 mL solution containing 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 μg/mL catalase, 5.0 mM sodium ascorbate, and 5 mM 2-(4-chlorophenylthio)acetic acid. O₂ consumption was measured as described in the materials and methods section. Note that the background rate has been removed for clarity.
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Figure 2.9. Effects of mercury competition with thiol for copper on O₂ consumption. The reaction was prepared as indicated in the Materials and Methods section for monitoring oxygen consumption. Upon initiation of the reaction with the addition of PAM (A), the reaction went to completion which is indicated by the slope of oxygen consumption matching that of the background rate prior to the addition of enzyme. The sample was then spiked with HgCl₂ to a final concentration of 5 µM (B). Oxygen consumption then resumed until 1.2 µM of additional oxygen had been consumed. Note that the background rate has been removed for clarity.
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Figure 2.11. PTAA and N-benzylglycine docked into the PHM crystal structure. (A) PTAA was docked into the PHM crystal structure (1SDW). The carboxylate forms a salt bridge with the R240 guanidino as expected. However, the absence of an amide hydrogen available for hydrogen bonding to the N316 oxygen allows for a great deal of movement and prevents proper positioning of the Cα-H for abstraction by an activated-oxygen species. (B) N-benzylglycine docked for comparison to illustrate proper hydrogen bonding and positioning. Non-amide hydrogens were omitted for clarity.
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Figure 2.14. L₃Cu¹⁺-thiophenolate system geometry optimized with the LanL2dz+ECP basis set.
Table 2.1. Steady-state kinetic constants for 2-(phenylthio)acetic acid and analogs

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>$K_{M,\text{app}}$ (mM)</th>
<th>$V_{\text{max,app}}$ (s$^{-1}$)</th>
<th>($V_{\text{MAX}}/K_M$)$_{\text{app}}$ (mM$^{-1}$ s$^{-1}$)</th>
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<tbody>
<tr>
<td>[(1-H-Indol-2-ylcarbonyl)thio]acetic acid</td>
<td><img src="image1" alt="Structure" /></td>
<td>2.3 ± 0.6</td>
<td>0.4 ± 0.01</td>
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<tr>
<td>2-(phenylthio)acetic acid</td>
<td><img src="image2" alt="Structure" /></td>
<td>0.95 ± 0.13</td>
<td>1.5 ± 0.2</td>
<td>1.6 ± 0.3</td>
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<tr>
<td>α-D,D-2-(phenylthio)acetic acid</td>
<td><img src="image3" alt="Structure" /></td>
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<td>2-(4-chlorophenylthio)acetic acid</td>
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<td>0.53 ± 0.11</td>
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<tr>
<td>2-(4-methylphenylthio)acetic acid</td>
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<td>4.2 ± 0.2</td>
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Table 2.2. PAM Inhibitors.

<table>
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<th>Name</th>
<th>Structure</th>
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</thead>
<tbody>
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<tr>
<td>2-(2-nitropheylthio)acetic acid</td>
<td><img src="image2" alt="Structure" /></td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>N-benzoyl-D-alanylthioglycolic acid</td>
<td><img src="image3" alt="Structure" /></td>
<td>0.89 ± 0.01</td>
</tr>
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</table>
Introduction

Peptidylglycine α-hydroxylating monooxygenase (PHM) is a copper-, oxygen- and ascorbate-dependent enzyme responsible for activating glycine-extended peptide prohormones through an oxidative cleavage yielding the corresponding peptide amide [1-6]. This reaction proceeds through the abstraction of hydrogen from the glycyl Cα followed by hydroxylation of said Cα and finally cleavage of the amide-Cα bond resulting in glyoxyate and the peptide amide. This bifunctional enzyme consists of the peptidylglycine α-hydroxylating monooxygenase (PHM) and the zinc-, calcium-, and iron-dependent peptidylglycine amidoglycolate lyase (PAL) domains, responsible for the hydrogen abstraction and hydroxylation, and the hydrolysis of the amide-Cα bond, respectively (Figure 3.1) [7, 8]. PHM and its sister enzyme, dopamin β-monooxygenase, have been studied extensively and are mechanistically similar[9]. The products of these reactions are essential signaling molecules which are stored in secretory granules and used for intercellular communication.

PAM activates several inflammatory hormones such as substance P. Previous studies have targeted PAM using mechanism-based inactivators to inhibit carrageenan-induced edema as well as all three phases of adjuvant-induced polyarthritis in rats [10]. Substance P is also found in the spinal fluid of fibromyalgia patients, making PAM a possible drug target for such inflammatory diseases. Genetically engineered mice and Drosophila lacking a functioning PHM gene generally die as embryos [11-13]. Thus, simply targeting PAM or PHM activity would be lethal. However, with advances in tissue-specific targeted drug delivery, this will become less of an obstacle.
Cinnamic acid and its derivatives are found in fruits, vegetables, and flowers. Cinnamic acid is an intermediate in the shikimate pathway, the pathway which links carbohydrate metabolism to the biosynthesis of aromatic compounds in plants. Recent studies on the utility of cinnamic acid derivatives as insulin releasing agents have proven certain ring-substituted cinnamic acids very effective in lowering plasma glucose levels [14, 15]. It has been suggested that cinnamic acid derivatives may be effective as treatment of diabetes mellitus for the regulation of blood glucose levels through the stimulation of insulin secretion [14].

Here we report on the investigation of several inactivators of PAM related to cinnamic acid. These compounds, whose derivatives are used as food and fragrance additives, are structurally similar to a known suicide substrate, 4-phenyl-3-butenoic acid[16]. Our results demonstrate that while the inactivation is turnover-dependent, the inactivator does not undergo any detectible chemistry as is typical of a suicide substrate. This finding merits further study into the effects of cinnamic acid inactivation of PAM in vivo as a potential drawback of using these molecules as diabetes therapeutics.

Materials and Methods

Materials

Cinnamic acid, 2-trifluorocinnamic acid, 3-(3-pyridyl)acrylic acid, phenylpropionic acid, 3,4-methylenedioxy cinnamic acid, N,N-dimethylaminocinnamic acid, maleamic acid, N-phenylmaleamic acid, Urocanic acid, 4-aminocinnamic acid, and perdeuterated cinnamic acid were from Sigma. Bovine catalase was from Worthington and 4-anilino-4-oxobut-2-enolic acid was from Enamine. Recombinant rat PAM was a gift from Unigene Laboratories, Inc. All other reagents were of the highest quality commercially available.
Synthesis

**Dansyl-4-aminocinnamic acid**

A solution of dansyl-chloride (400 mg, 1.5 mmol) in a minimal dry pyridine (~2 mL) was added dropwise to a N₂-purged solution of 4-aminocinnamic acid (500 mg, 3.1 mmol) in 50 mL of dry pyridine at 60 °C. After 24 hours, the reaction was diluted then extracted with Et₂O (100 mL x 3), yielding a yellow oil. Crystallization of the final dansyl-4-aminocinnamic acid was done with methanol/ddH₂O (285 mg, 48%).

**3-phenyloxirane-2-carboxylic acid**

Cinnamic acid (5 g, 34 mmol) dissolved in a 30 mM NaOH(aq) (~100 mL) was combined with an oxone (30 g, 98 mmol) solution in H₂O (100 mL). A constant pH of six was maintained over six hours through the addition of NaOH then stirred for an additional 16 hours. Acid extraction (pH 1) of the reaction was performed with Et₂O (500 mL) by vigorously stirring the bi-phasic solution for ~2 hours. The organic layer was then separated and removed under reduced pressure. The resulting residue was crystallized with ethanol/H₂O (1.6 gm, 29%).

**In silico ligand docking**

The crystal structure for reduced peptidylglycine α-hydroxylating monooxygenase (PHM) was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/, 1SDW) [17]. 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 hydrogens were added using *Maestro* (www.schrodinger.com). Further receptor refinements were carried out utilizing ProteinPrep from within *Maestro*. Investigation
of inhibitor binding modes were performed using Glide[18] and Qsite[19] 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 [20, 21].

**DFT investigation of Ca-H dissociation energies**

The bond dissociation energies of Ca-H for all cinnamic acid analogs were calculated using Jaguar [22]. DFT 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 Ca-H bond was then increased from 1.08 – 3.0 Å while holding the molecule rigid (Figure 3.2), and single point energies were calculated.

**Inhibition of O2 consumption from N-acetylglycine by cinnamic acid**

Reactions at 37.0 ± 0.1 °C were initiated by the addition of PAM (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(NO₃)₂, 5.0 mM sodium ascorbate, 1.0 – 45 mM N-acetylglycine, and 0–9 mM cinnamic acid. Initial rates were measured by following the PAM-dependent consumption of O₂ using a Yellow Springs Instrument Model 5300 oxygen monitor.

**Inactivation of PAM by cinnamic acid**

Inactivation reactions 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(NO₃)₂, 5.0 mM sodium ascorbate, and 0-3 mM cinnamic acid were initiated by the addition of enzyme and incubated at 37 °C. Aliquots of 15 µL were withdrawn at various intervals and diluted into 2.0
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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(NO₃)₂, 5.0 mM sodium ascorbate, and 20 mM N-acetylglycine and monitored for O₂ consumption.

Reversibility of inactivation

An inactivation reaction of 250 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(NO₃)₂, 5.0 mM sodium ascorbate, and 7 mM cinnamic acid was initiated by the addition of enzyme and incubated at 37 °C for 2 hours. The reaction was then extensively dialyzed and concentrated to 50 µL. The concentrate 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, a 100 µL aliquot was removed, added to a vial containing 20 µL of 6% (v/v) TFA to terminate the reaction, and the concentration of glyoxylate formed measured in the acidified samples to test for the recovery of activity.

PAM labeling by dansyl-4-aminocinnamic acid

Covalent modification of PAM was investigated by using dansyl-4-aminocinnamic acid, a fluorescent molecule, as an inactivator. A 0.5 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 µg/ml bovine catalase, 1.0 µM Cu(NO₃)₂, 5.0 mM sodium ascorbate, and 1.0 mM dansyl-4-aminocinnamic acid was initiated by the addition of 50 µg of enzyme and incubated at 37 °C for 3 hours. 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 hours changing the dialysis buffer every hour. The reaction mixture was then concentrated by ultra-filtration to ~100 µL. This sample was then
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trypsin digested and the peptide fragments were analyzed by RP-HPLC utilizing a fluorescence detector.

*PAM labeling by $^{14}$C-cinnamic acid*

Enzyme modification was also examined using radio-labeled cinnamic acid. A 250 µL reaction 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$_3$)$_2$, 5.0 mM sodium ascorbate, and 3 µCi of $^{14}$C-cinnamic acid was initiated by the addition of 18 – 52 µg of PAM. The reaction was allowed to incubate at 37 °C for 3 hours before ultra-filtration was performed. The reaction was then washed with 200 µL 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 poly(ethylene glycol) to remove excess non-enzyme bound radio-labeled cinnamic acid. Counts per minute were then compared using a scintillation counter.

*PAM modification through inactivation by cinnamic acid*

Modification of the PAM active site by the reactive Cu/O species was also investigated as a possible means of cinnamic acid mediated inactivation. A 100 µL 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 µM Cu(NO$_3$)$_2$, 5.0 mM sodium ascorbate, and 3 mM cinnamic acid was initiated with 20 µg of PAM and incubated at 37 °C for 12 hours. 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 hours and the volume reduced to ~40 µL by ultra-filtration. The enzyme was then analyzed by MALDI-TOF against control for any modification. The reaction was then repeated as previously stated.
followed by trypsin digestion. The reaction was then analyzed by LC/MS, LC/MS/MS, and MALDI-TOF.

**PAL inactivation assay**

To determine if the cinnamic acid was also inactivating PAL activity by PAM, initial rates of glyoxylate formation from α-hydroxyhippurate, a known PAL substrate, were monitored. Reactions of 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 cinnamic acid or analog equal to 5-times $K_i$ of PHM-related inhibition for that particular inhibitor were incubated for 2 hours. 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 minute intervals from 10 to 60 min., a 100 µL aliquot was removed, added to a vial containing 20 µL of 6% (v/v) TFA to terminate the reaction, and the concentration of glyoxylate formed measured in the acidified samples.

Glyoxylate was determined by the spectrophotometric method of Christman *et al.* [23] as modified by Katopodis and May [24]. Standard curves of [glyoxylate] vs. $A_{320}$ were constructed in the appropriate buffers using a glyoxylate solution that had been calibrated by measuring the glyoxylate-dependent oxidation of NADH ($\Delta e_{340} = 6.22 \times 10^3$ M$^{-1}$ cm$^{-1}$) as catalyzed by lactate dehydrogenase.

**Analysis of steady-state kinetic data**

Initial rate studies that resulted in competitive inhibition were also analyzed by Kaleidagraph® fit of $v$ vs. [S] as a function of inhibitor concentration to equation 1.
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\[ \nu = \frac{V_{\text{MAX,app}} [S]}{(K_{\text{M,app}} \left(1 + \frac{[I]}{K_{i,s}}\right) + [S])} \]  

(1)

Where \( K_{\text{M,app}} \) is the apparent Michaelis constant for the oxidizable substrate at fixed [ascorbate] and ambient [O\(_2\)] concentrations and \( V_{\text{MAX,app}} \) is the apparent maximum initial velocity at saturating [S].

Inactivation kinetics were analyzed by Kaleidagraph® fit of 1/\( k_{\text{obs}} \) vs 1/[I] to equation 2.

\[ \frac{1}{k_{\text{obs}}} = \frac{1}{k_{\text{inact}}} + \frac{K_{I}}{k_{\text{inact}} [I]} \]  

(2)

Where \( k_{\text{obs}} \) is the observed rate of inactivation, and \( k_{\text{inact}} \) is the intrinsic rate of inactivation of enzyme.

**Results**

*Cinnamic acid and analogs as inhibitors of PAM*

Cinnamic acid and several analogs were initially investigated as small molecule inhibitors of PAM. Cinnamic acid was shown to inhibit the consumption of O\(_2\) from N-acetylglycine in a competitive manner (Figure 3.3) yielding the steady-state kinetic values as described by equation 1 (Table 3.1). All cinnamic acid analogs were assumed competitive and also analyzed according to equation 1.

*Inactivation of PAM by cinnamic acid*

Cinnamic acid was investigated as an inactivator of PAM by the dilution method, a common method for determining the kinetic parameters of time-dependent inactivators [25].
Inactivation experiments performed with cinnamic acid indicated that it is a time-dependent inactivator of PAM (Figure 3.4). The inactivation was pseudo first-order and concentration dependent, as well as \( \text{O}_2^- \) and ascorbate-dependent (Figure 3.5). Substrate was also found to protect against inactivation of PAM by cinnamic acid. Perdeuterated cinnamic acid was also investigated in search of an isotope effect on \( k_{\text{inact}} \) as evidence for H-abstraction, though one was not observed (Table 3.2).

**Inactivation of PAL activity**

Cinnamic acid and analogs were tested as PAL inactivators as well to investigate whether or not the PAM activation occurring was specific to the PHM domain. The experiment indicates that PAL is completely unaffected by cinnamic acid and the various analogs tested and retained 100% activity when compared to the control reaction (Figure 3.6).

**Irreversible inactivation**

Cinnamic acid was able to irreversibly inactivate PAM. Extensive dialysis of a reaction mixture incubated with cinnamic acid yielded dead enzyme incapable of producing glyoxylate from hippuric acid (Figure 3.7).

**Non-labeled PAM**

Multiple experiments were performed in an attempt to verify covalent linkage of cinnamic acid to PAM. Both the fluorescent labeling with dansyl-4-aminocinnamic acid, and the radio labeling experiments indicated that cinnamic acid was not covalently linking to the enzyme. Multiple methods of mass analysis were performed in an attempt to elucidate any auto-oxidation or hydroxylation of the PAM active site as has been hypothesized in earlier studies.
with 4-phenyl-3-butenoic acid [26]. However, we were unable to detect any modification of the enzyme after exhausting all efforts and resources (Figure 3.8).

*Docking of cinnamic acid and analogs*

Docking of the investigated compounds yielded poses in agreement with the competitive nature of inhibition observed experimentally. The compounds bind forming a salt bridge between the carboxy terminus and the guanidino group of R240 as has been previously shown with glycine-extended substrates [27]. This interaction aligns the Cα-H in close proximity to CuM (Figure 3.9). However, the lack of glycyl amide hydrogen to H-bond with the N316 oxygen coupled with the compounds’ small size preventing any interaction with the nearby hydrophobic pocket would allow for a great deal of movement within the active site making hydrogen abstraction difficult.

*Computation results for bond energy*

The *ab initio* calculations performed to estimate the bond dissociation energies of the Cα-H bond for cinnamic acid and the investigated analogs indicate that more than 110 kcal/mol are needed (Table 3.3).

*Discussion*

Cinnamic acid is a structural analogue of 4-phenyl-3-butenoic acid (PBA), a well-documented mechanism-based, irreversible inhibitor (inactivator) of the PHM domain as both molecules contain an olefin moiety [16, 24]. While PBA has been suggested to be hydroxylated at both the α and γ positions by PHM [26], none of the compounds tested within this study displayed oxygen consumption when screened for activity even though inactivation was O₂- and
ascorbate-dependent. Further evidence that hydroxylation was absent with cinnamic acid was the lack of a deuterium kinetic isotope effect for $k_{\text{inact}}$ or $k_{\text{inact}}/K_I$. Although the presence of an isotope effect would give evidence for hydrogen abstraction from the $\text{C}\alpha$ of cinnamic acid, absence may suggest that the magnitude of $Dk_{\text{inact}}$ may be suppressed by much faster steps in the mechanism. The lack of PHM inactivation with 3-phenyloxirane-2-carboxylic acid suggests that C-H cleavage is required to generate the inactivating olefin intermediate species in the hydroxylation pathway. The C-H bond dissociation energies, displayed in Table 3.3, display enthalpies for each conjugated species to be very close to each other (a difference of only 13 kcal/mole). Therefore, if the inactivation species were ultimately dependent only on a C-H cleavage step, 3-phenyloxirane-2-carboxylic acid would be expected to inactivate PHM. This suggests that the inactivation is dependent on the presence of an olefin while the lack of inactivation by phenylpropionic acid, the alkyne derivative, suggests that the $\text{C}\alpha$-H is also a prerequisite for inactivation. This may suggest that the inactivating species is an intermediate along the hydroxylation pathway after the abstraction of the $\text{C}\alpha$-H and that the inactivation is dependent on the olefin. This species may be a vinyl radical present on the $\text{C}\alpha$ as this radical would not delocalize.

The instrumental nature of the olefin radical generated upon hydrogen abstraction further suggest that dynamical freedom of this small inactivator may also be important to this inactivation mechanism. The modeling performed suggests that there are few interactions to hold cinnamic acid in the active site other than the salt-bridge with R240. This is also reflected in the poor $K_I$ value for cinnamic acid (3.6 mM, Table 1). This may be directly attributed to the lack of amide hydrogen to anchor the $\text{C}\alpha$-H and properly position it for abstraction along with the inability of the phenyl ring to bury itself into the hydrophobic pocket to further stabilize the
molecule. This results in a significant increase in the conformational sampling and a decrease in
the probability of proper wave function overlap between donor and acceptor [28]. Upon
successful hydrogen abstraction, the radical character generated can be shared between α and β
carbons. Assuming the Cu-alkoxide intermediate, hydroxylation of such a mobile species with
delocalized radical character would prove difficult. Thus, the reactive cinnamic acid species
may leave the active site allowing for hydroxylation to occur by the addition of water to the
radical intermediate. This would leave the activated Cu-oxo species to either modify the enzyme
in a way undetectable by mass analysis or perhaps linger in a trapped dynamic state unable to
return to the free-enzyme state (Figure 3.10).

Conclusion

In conclusion, we have demonstrated that potential diabetes drug candidates are capable
of PAM inactivation. Because of the importance of PAM in the biosynthesis of amidated peptide
hormones, these results are of great pharmacological significance. As the development of small-
molecule insulin secretion drugs with the α,β olefin moiety progresses, special attention must be
paid to the possible inactivation of PAM. These findings are also therapeutically relevant as
PAM is over-expressed in both small cell lung cancer and prostate cancer [29-31] making it a
possible drug target.
Chapter Three: Cinnamic Acid Derivatives as Inactivators of PAM

References


Chapter Three: Cinnamic Acid Derivatives as Inactivators of PAM


Figures and Tables

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Figure 3.1. PAM catalytic scheme.

\[
\begin{align*}
\text{PHM} & \\
\text{PAL} & \\
\end{align*}
\]
Figure 3.2. Illustration of carbon-hydrogen bond elongation for the DFT calculation of bond dissociation energies.
Figure 3.3. Inhibition of PAM by cinnamic acid. Initial rates were determined at 37 °C as described in the Materials and Methods section. The points are experimentally determined initial rates. The lines were computer fit to the data using equation 1.
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### Table 3.1: Inhibition constants calculated from computer fit of data to equation 1.

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<th>Structure</th>
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Chapter Three: Cinnamic Acid Derivatives as Inactivators of PAM

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Table 3.2. Inactivation constants calculated by Kitz-Wilson analysis using the dilution assay method as outline in the materials and methods section.

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<th>Compound</th>
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<th>$k_{\text{inact}}/K_1$ (mM$^{-1}$ min$^{-1}$)</th>
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<td>Cinnamic acid</td>
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<td>0.04 ± 0.006</td>
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<td>N,N-dimethylaminocinnamic acid</td>
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<td>0.06 ± 0.01</td>
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<td>Dansyl-4-aminocinnamic acid</td>
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<td>Perdeuterated cinnamic acid</td>
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<td>0.04 ± 0.004</td>
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Table 3.3. Calculated bond dissociation energies of selected cinnamate analogs by DFT/6-31G*.

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Chapter Four: PAM Inactivation by Phenylbutenoates: 
Evidence for a radical intermediate

Introduction

Peptidylglycine α-amidating monooxygenase (PAM) is a bifunctional metalloenzyme responsible for the conversion of glycine-extended peptide prohormones to the active hormone, peptide-amide [1, 2]. Catalysis proceeds through the abstraction of the glycyl Cα hydrogen via a reactive Cu-oxo species followed by hydroxylation, and finally the cleavage of the N-Cα bond to yield glyoxylate and the peptide-amide. Two domains in PAM are responsible for this chemistry. The peptidylglycine α-hydroxylating monooxygenase (PHM) domain is responsible for the hydrogen abstraction and subsequent hydroxylation while the peptidylglycine amidoglycolate lyase (PAL) domain is responsible for the cleavage of the N-Cα bond (Figure 4.1) [3]. PHM activity is copper-, ascorbate-, and O₂-dependent while PAL catalysis is zinc-, calcium-, and iron-dependent [4-9].

PHM and its sister enzyme dopamine β-monoxygenase (DβM) are mechanistically related. Both PHM and DβM produce essential signaling molecules which are stored in secretory granules and used for intercellular communication [10]. They catalyze very similar reactions utilizing a reactive copper-oxygen species to hydroxylate their very different respective substrates (Figure 4.2). PHM and DβM are essential enzymes as genetically engineered mice and Drosophila melanogaster lacking a functional PHM or DβM gene generally die as embryos [11-13]. PAM/PHM and DβM have been the focus of much mechanistic study. Many of the proposed mechanistic schemes involve a substrate free radical intermediate (Figure 4.3). All of the work providing evidence for this radical intermediate has been performed on DβM. Several studies in particular have provided evidence for a substrate radical mechanism in DβM [14-17].
No such studies have been performed with PHM though the results of the DβM studies have been applied towards the elucidation of both mechanisms.

Here we report experiments that provide evidence for a radical intermediate in PAM catalysis. Ring-substituted suicide substrates are used to probe electronic effects to define the nature of the reactive intermediate. The results provide evidence for a radical mechanism and are in agreement with previous studies performed on DβM.

Materials and Methods

Materials

4-Phenyl-3-butenoic acid and N-acetylglycine were from Sigma Aldrich. 4-(4-chlorophenyl)-3-butenoic acid, 4-(4-methoxyphenyl)-3-butenoic acid, 4-(3-chlorophenyl)-3-butenoic acid, and 4-(3-methoxyphenyl)-3-butenoic acid were a gift from Dr. John Vederas. Bovine catalase was from Worthington. Recombinant rat PAM was a gift from Unigene Laboratories, Inc. All other reagents were of the highest quality commercially available.

In silico docking

The crystal structure for reduced peptidylglycine α-hydroxylating monooxygenase (PHM) was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/, 1SDW) [18]. All species deemed superfluous for ligand binding were removed from the pdb file (nickel, water, glycerol, and substrate). Formal charges for enzyme-bound copper ions were set as 1, bond orders were corrected, and hydrogens were added using Maestro (www.schrodinger.com). Further receptor refinements were carried out utilizing ProteinPrep from within Maestro. Investigation of the phenylbutenoate binding modes were performed using Glide[19] and
Qsite[20] 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 [21, 22].

**Radical stabilization energies**

To investigate the effects of the substituents on the relative stability of the $C\alpha$ radical, *ab initio* molecular orbital theory and density functional theory calculations were performed using PCGAMESS. The geometries of each phenylbutenoate and the corresponding radical along with methane and the methane radical were determined at the B3LYP/6-31G(d) level. Single point energy calculations were then carried out on the optimized structures at the MP2 level with 6-311+G(2df,p) basis sets. Radical stabilization energies of PBA radicals were calculated as energies of the following reaction:

$$ \text{CH}_4 + \text{RHC}^*\text{COOH} \rightarrow \text{C}^*\text{H}_3 + \text{RCH}_2\text{COOH} $$  \hspace{1cm} (1)

Thus, the difference between the C—H bond dissociation energy of methane and the C—H bond dissociation energy in the phenylbutenoates.

$$ \text{RSE}(\text{RHC}^*\text{COOH}) = \text{BDE}(\text{CH}_4) - \text{BDE}(\text{RCH}_2\text{COOH}) $$ \hspace{1cm} (2)

A positive radical stabilization energy value would then indicate that the radical is stable relative to the methane radical compared with the corresponding closed-shell systems.

**Inactivation of PAM by 4-phenyl-3-butenoates at ambient $O_2$**

Inactivation reactions of 50 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$_3$)$_2$, 5.0 mM sodium ascorbate, and various concentrations of phenylbutenoates were initiated by the addition of enzyme and incubated at 37 °C. Aliquots of 20 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$_3$)$_2$, 5.0 mM sodium ascorbate, and 20 mM N-acetylglycine. The residual activity was thus measured by the rate of oxygen consumption. Because of the high potency of the phenylbutenoates, the inactivation reactions were not performed continuously due to the down time required to prepare the next reaction mixture within the oxygen electrode chamber. Thus, a separate reaction was required for each time-point at each concentration.

\textit{Inactivation of PAM by 4-phenyl-3-butenoates at variable O$_2$ concentrations}

Inactivation stock reactions of 200 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$_3$)$_2$, 5.0 mM sodium ascorbate, and various concentrations of phenylbutenoates were incubated at 37 °C for 10 minutes in septum sealed tubes. The rubber septum was then pierced with a 25 gauge needle as a pressure release and a second needle attached to non-permeable rubber tubing. Through this tubing, the desired concentration of O$_2$ was delivered by means of an ACS certified O$_2$:N$_2$ mixture. The delivery needle was submerged in the reaction solution to “bubble through” the gas mixture. Bubbling of the gas mixture through the reaction solution prevented the evaporation of reaction mixture that was noted when blowing over the gas mixture while stirring the solution. While the total volume change related to this evaporation was small in absolute terms (20 – 40 µL), it was significant relative to the total reaction volume making the inactivator concentrations unreliable. The thin gauge needle allowed for sufficient aeration of the samples providing both accurate concentrations of O$_2$. After streaming the gas mixture through the reaction sample for 5 minutes, one empty reaction tube was prepared with rubber septum for each time point to be taken at that particular inactivator concentration. A typical inactivation study by the dilution method is classically a continuous assay. However, the time
required to prepare the oxygen electrode in between time points rendered a continuous assay method impossible. Thus, each time point required the preparation of a new reaction mixture and the incubation was repeated from time = 0. Each empty reaction tube with septum was then purged with the desired O2:N2 reaction mixture and the needles removed. 30 µL of the stock reaction was then placed into each purged tube with a 25 gauge needle and syringe. Each reaction was then initiated with enzyme and incubated for the desired length of time before removing a 20 µL aliquot and initiating a 2.0 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 µg/ml bovine catalase, 1.0 µM Cu(NO3)2, 5.0 mM sodium ascorbate, and 20 mM N-acetylglycine and monitoring O2 consumption to examine residual enzyme activity.

Reversibility of Inactivation

An inactivation reaction 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(NO3)2, 5.0 mM sodium ascorbate, and 100 µM 4-phenyl-3-butenoic acid was initiated by the addition of enzyme and incubated at 37 °C for 2 hours. The reaction was then extensively dialyzed and concentrated to ~50 µL. The concentrate 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, a 100 µL aliquot was removed, added to a vial containing 20 µL of 6% (v/v) TFA to terminate the reaction, and the concentration of glyoxylate formed measured in the acidified samples to test for the recovery of activity.
Inactivation of PAL activity by 4-phenyl-3-butenoic acid

To determine if 4-phenyl-3-butenoic acid was also inactivating PAL activity of PAM, initial rates of glyoxylate formation from α-hydroxyhippurate, a known PAL substrate, were monitored. Reactions of 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 100 µM 4-phenyl-3-butenoic acid was incubated for 2 hours. 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 various time intervals, a 100 µL aliquot was removed, added to a vial containing 20 µL of 6% (v/v) TFA to terminate the reaction, and the concentration of glyoxylate formed measured in the acidified samples.

Determination of partition ratios

To determine the partition ratio for product formation versus enzyme inactivation, a 2.0 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 µg/ml bovine catalase, 1.0 µM Cu(NO₃)₂, 5.0 mM sodium ascorbate, and 1 mM of the desired 4-phenyl-3-butenoic acid derivative. Oxygen consumption was then monitored by oxygen electrode to monitor hydroxylation activity.

Determination of glyoxylate concentration

Glyoxylate was determined by the spectrophotometric method of Christman et al. [23] as modified by Katopodis and May [24]. Standard curves of [glyoxylate] vs. A₅₂₀ were constructed in the appropriate buffers using a glyoxylate solution that had been calibrated by measuring the glyoxylate-dependent oxidation of NADH (Δε₅₄₀ = 6.22 x 10⁻³ M⁻¹ cm⁻¹) as catalyzed by lactate dehydrogenase.
Investigation of inactivation through PAM modification

Modification of the PAM active site by the activated Cu/O species was investigated as a possible means of phenylbutenoate mediated inactivation in addition to covalent labeling by phenylbutenoate. A 100 µL 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 µM Cu(NO₃)₂, 5.0 mM sodium ascorbate, and 500 µM 4-phenyl-3-butenoic acid was initiated with 20 µg of PAM and incubated at 37 °C for 12 hours. 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 hours and the volume reduced to ~40 µL by ultrafiltration. The enzyme was then analyzed by MALDI-TOF against control for any modification. The reaction was then repeated as previously stated followed by trypsin digestion. The reaction was then analyzed by both LC/MS and MALDI-TOF.

Analysis of kinetic data

Inactivation kinetics were analyzed by Kaleidagraph fit of $1/k_{obs}$ vs $1/[I]$ to equation 3.

$$\frac{1}{k_{obs}} = \frac{1}{k_{inact}} + \frac{K_I}{k_{inact} [I]} \quad (3)$$

where $k_{obs}$ is the observed rate of inactivation, and $k_{inact}$ is the intrinsic rate of inactivation of enzyme.

Results

Molecular modeling

The phenylbutenoates of interest were docked into the reduced PHM crystal structure. As expected, the terminal carboxylate formed a salt-bridge with R240 (Figure 4.4). The Cα-H is
in close proximity to the catalytic active site copper, \( \text{Cu}_M \) (Figure 4.5). The phenylbutenoates are not large enough, however, for the phenyl groups to bury themselves into the hydrophobic pocket to restrict movement of the molecule. The small size in addition to the lack of an amide hydrogen needed for hydrogen bonding to N316 to further limit ligand mobility within the active site allows for a great deal of movement of the phenylbutenoates once within the active site. Molecular dynamics simulations performed with the docked 4-phenyl-3-butenoate using NAMD 2.6 demonstrate that there is, indeed, a great deal of mobility for these small compounds without the requisite interactions present when peptide-gly substrates are bound to PAM.

**Partition ratios**

The partition ratios for the ring-substituted phenylbutenoates were determined by measuring the total oxygen consumed during inactivation as a stoichiometric amount is \( \text{O}_2 \) is consumed during hydroxylation. Thus, upon complete inactivation the amount of \( \text{O}_2 \) consumed is equivalent to the amount of hydroxylated phenylbutenoate produced. All phenylbutenoates investigated are efficient inactivators with the most efficient giving a partition ratio of 18 turnovers per inactivation (Table 4.1).

**Inactivation of PAM by phenylbutenoates**

Several ring-substituted phenylbutenoates were investigated as inactivators of PAM by the dilution method, a common method for determining the kinetic parameters of time-dependent inactivators [25]. Inactivation experiments performed with these analogs indicated that they are indeed time-dependent inactivators of PAM (Figure 4.6). The inactivation was pseudo first-order and concentration dependent, as well as \( \text{O}_2 \)- and ascorbate-dependent. Substrate was found to protect against inactivation of PAM by 4-phenyl-3-butenoic acid (Figure 4.7). The inactivation was found to be irreversible. Extensive dialysis of a reaction mixture incubated with
4-phenyl-3-butenoic acid yielded dead enzyme incapable of producing glyoxylate from hippuric acid and no oxygen consumption was observed when monitored with the oxygen electrode. The kinetic parameters for inactivation under ambient conditions are listed in Table 4.2.

The ring-substituted phenylbutenoates were then further examined to investigate the effects of substitution on the partition ratio, $V_{\text{max}}/k_{\text{inact}}$, and the kinetics of inactivation. A double reciprocal plot of $1/k_{\text{obs}}$ vs. $1/[PBA]$ at fixed O$_2$ concentrations yields a pattern of intersecting lines

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k_{\text{inact}}} + \frac{K_{\text{PBA}}}{k_{\text{inact}}[PBA]} + \frac{K_{O_2}}{k_{\text{inact}}[O_2]} + \frac{K_{(d,PBA)K_{O_2}}}{k_{\text{inact}}[PBA][O_2]}$$  \hspace{1cm} (3)

with a slope of:

$$\frac{K_{\text{PBA}}}{k_{\text{inact}}} + \frac{K_{d,PBA}K_{O_2}}{k_{\text{inact}}[O_2]}$$  \hspace{1cm} (4)

and the ordinate intercept of:

$$\frac{1}{k_{\text{inact}}} + \frac{K_{O_2}}{k_{\text{inact}}[O_2]}$$  \hspace{1cm} (5)

A replot of the intercept vs. $1/[O_2]$ yields a straight line. From the inactivation kinetics at varying concentrations of O$_2$, a linear free energy plot of $k_{\text{inact}}/K_{O_2}$ values can be constructed as a function of electron-withdrawing or electron-donating ability of the substituent (Figure 4.8).

**Inactivation of PAL activity**

4-Phenyl-3-butenolic acid was tested as a PAL inactivator as well to investigate whether or not the PAM inactivation occurring was specific to the PHM domain. Glyoxylate was readily formed from $\alpha$-hydroxyhippurate, a known PAL substrate. The experiment indicates that PAL is
completely unaffected by 4-phenyl-3-butenoic acid and retained 100% activity when compared to the control reaction (Figure 4.9).

*Non-labeled PAM*

Multiple methods of mass analysis were performed in an attempt to elucidate any auto-oxidation or hydroxylation of the PAM active site as has been hypothesized in earlier studies with 4-phenyl-3-butenoic acid [26]. While the hydroxylated product was detected by LC/MS, we were unable to detect any modification of the enzyme by MALDI-TOF, or LC/MS and LC/MS/MS on trypsin digested samples (Figure 4.10).

*Radical stabilization energies*

The radical stabilization energies calculated from the isodesmic reaction discussed previously indicate that several of the phenylbutenoates are capable of forming extremely stable radicals when compared to the methane radical [27]. While the radical stabilities are relative and not absolute, the data indicates that the 4-(4-chlorophenyl)-3-butenoic acid and 4-(4-methoxyphenyl)-3-butenoic acid derivatives are greater than an order of magnitude more stable than the other phenylbutenoates analogs investigated (Table 4.3). These two very stable derivatives also had the highest partition ratios. Thus, there exists a direct correlation between radical stability and the ability of PHM to hydroxylate the phenylbutenoates after hydrogen abstraction.

*Discussion*

The phenylbutenoates investigated meet the criteria for mechanism-based inhibitors of PAM. The inactivation is both ascorbate- and O$_2$- dependent. The phenylbutenoates all act as suicide substrates under turnover conditions inactivating the enzyme. The inactivation is protected against by the presence of substrate.
Chapter Four: PAM Inactivation by Phenylbutenoates: Evidence for a radical intermediate

The experiments performed were designed to give evidence of a radical intermediate being formed during PAM catalysis. To accomplish this, the kinetics of inactivation and the partition ratios were examined using phenylbutenoates with substituents on the phenyl ring of different electron-donating and electron-withdrawing effects. The steady-state kinetics of PAM with PBA can be described as

\[
v = V + \frac{V[\text{Asc}]}{K_{\text{Asc}}} + \frac{V[P\text{BA}]}{K_{P\text{BA}}} + \frac{V[O_2]}{K_{O_2}} + \frac{V[P\text{BA}]O_2}{K_{P\text{BA}}K_{O_2}}\]

A minimal mechanism accounting for catalysis and inactivation is described as

\[
\begin{align*}
E_{\text{Cu}(II)} & \quad \text{k}_1 [\text{Asc}] \quad E_{\text{Cu}(II)} \quad \text{k}_2 \quad E_{\text{Cu}(II)} \quad \text{k}_3 \quad E_{\text{Cu}(II)} & \quad \text{k}_4 [\text{PBA}] \quad E_{\text{Cu}(II)} \quad \text{k}_5 \quad E_{\text{Cu}(II)} \quad \text{k}_6 \quad E_{\text{Cu}(II)} \quad \text{k}_7 \quad O_2 \quad E_{\text{Cu}(II)} & \quad \text{k}_8 \quad E_{\text{Cu}(II)} \quad \text{k}_9 \quad E_{\text{Cu}(II)} \quad \text{k}_{10} \quad E_{\text{Cu}(II)} \quad \text{k}_{11} \quad E_{\text{Cu}(II)} \quad \text{k}_{12} \quad E_{\text{Cu}(II)} \quad \text{k}_{13} \quad E_{\text{Cu}(II)} \quad \text{k}_{14} \quad E_{\text{Cu}(II)} \quad \text{k}_{15} \quad E_{\text{Cu}(II)}
\end{align*}
\]

For this inactivation scheme, the rate constant for the catalytic steps is \(k_9\). \(V/K_{O_2}\) is then the kinetic parameter that contains the catalytic step and would equal \(k_7k_9/(k_8 + k_9)\). The partition ratio for inactivation for a mechanism-based inhibitor is \(V_{\text{max}}/k_{\text{inact}}\). Thus, one can arrive at \(V/K_{O_2}\) by simply multiplying the partition ratio by the inactivation kinetic data; this allows the attainment of steady-state kinetic data when such data cannot be obtained directly. Therefore, we have multiplied the partition ratios by the respective \(k_{\text{inact}}/K_{O_2}\) values of the phenylbutenoates to calculate the \(V/K_{O_2}\) values. A free energy plot was then created with these values and compared to various sigma constants for each substituent (Figure 4.11). The different sigma constants differ in that they are based on different standard reaction series. When compared to \(\sigma^+\), a \(\rho\) value of -1.03 was obtained.
Similar studies have previously been performed on D-amino acid oxidase. This flavoprotein was found to go through a carbanion intermediate [28]. The $\rho$ value obtained when $V_{\text{max}}$ was measured for a series of ring-substituted phenylglycines was 5.44 [29]. This finding suggests that PAM does not proceed through a carbanion mechanism. That PAM proceeds via a radical mechanism is consistent with the data which shows a small absolute magnitude for the value of $\rho$ and also the correlation with $\sigma^+$, results characteristic of a radical mechanism [14]. Studies on styrene derivatives involving allylic hydrogen abstraction through a radical mechanism have demonstrated similar results, finding a Hammett slope $\rho = -0.82$ [30].

The partition ratios and relative radical stabilities calculated using *ab initio* methods also support a radical mechanism. The partition ratios are all within an order of magnitude. The agreement seen between the radical stability of the two most stable phenylbutenoates calculated and that they are also the two ligands with the highest partition ratios provides further evidence that catalysis proceeds via a radical mechanism. The increased stability for the radical on the C$\alpha$ allows for a greater ease in hydroxylation by the PHM domain in PAM. This leads to more turnovers before inactivation occurs.

**Conclusion**

In conclusion, we report here findings that suggest PAM catalysis occurs through a radical mechanism. Other studies have shown that two hydroxylation products are formed during phenylbutenoate catalysis leading to both the alpha and gamma hydroxylated derivatives [26]. The most likely mechanism by which this hydroxylation may proceed is through the resonance of a radical generated at the alpha position during hydrogen abstraction (Figure 4.12). The dynamical freedom allowed the phenylbutenoate from the lack of amide-hydrogen to N316 hydrogen bonding, an interaction present in the peptide-gly substrates (Figure 4.13), has been
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hypothesized to cause the lack of absolute stereospecificity in hydroxylation of the 2-position observed in earlier studies [26]. This mobility coupled with the resonance of the radical may reduce the probability of hydroxylation at the 2-position allowing for release of the PBA radical intermediate leaving an activated oxygen at Cu$_M$. This would also allow for autoxidation of the active site resulting in inactive enzyme. Water would then be the hydroxylating species for the 4-OH-PBA species.
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Table 4.1. Partition ratios for ring-substituted 4-phenyl-3-butenoic acids

<table>
<thead>
<tr>
<th>Name</th>
<th>Partition Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-phenyl-3-butenoic acid</td>
<td>85</td>
</tr>
<tr>
<td>4-(4-chlorophenyl)-3-butenoic acid</td>
<td>94</td>
</tr>
<tr>
<td>4-(3-chlorophenyl)-3-butenoic acid</td>
<td>35</td>
</tr>
<tr>
<td>4-(4-methoxyphenyl)-3-butenoic acid</td>
<td>143</td>
</tr>
<tr>
<td>4-(3-methoxyphenyl)-3-butenoic acid</td>
<td>18</td>
</tr>
</tbody>
</table>
Table 4.2. The kinetic parameters for the inactivation of PHM by ring-substituted 4-phenyl-3-butenoic acids.

<table>
<thead>
<tr>
<th>Name</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$K_1$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-phenyl-3-butenoic acid</td>
<td>4.18</td>
<td>0.004</td>
</tr>
<tr>
<td>4-(4-chlorophenyl)-3-butenoic acid</td>
<td>13.6</td>
<td>0.98</td>
</tr>
<tr>
<td>4-(3-chlorophenyl)-3-butenoic acid</td>
<td>1.18</td>
<td>1.45</td>
</tr>
<tr>
<td>4-(4-methoxyphenyl)-3-butenoic acid</td>
<td>0.22</td>
<td>0.06</td>
</tr>
<tr>
<td>4-(3-methoxyphenyl)-3-butenoic acid</td>
<td>0.10</td>
<td>0.04</td>
</tr>
</tbody>
</table>
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Table 4.3. Radical stabilization energies for ring-substituted 4-phenyl-3-butenoic acids.

<table>
<thead>
<tr>
<th>Name</th>
<th>RSE (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-phenyl-3-butenoic acid</td>
<td>47.6</td>
</tr>
<tr>
<td>4-(4-chlorophenyl)-3-butenoic acid</td>
<td>1480</td>
</tr>
<tr>
<td>4-(3-chlorophenyl)-3-butenoic acid</td>
<td>53.9</td>
</tr>
<tr>
<td>4-(4-methoxyphenyl)-3-butenoic acid</td>
<td>1440</td>
</tr>
<tr>
<td>4-(3-methoxyphenyl)-3-butenoic acid</td>
<td>49.5</td>
</tr>
</tbody>
</table>
Introduction

Peptidylglycine α-amidating monooxygenase (PAM) is a copper- and zinc-dependent, bifunctional enzyme that catalyzes the cleavage of glycine-extended peptides to the corresponding amides and glyoxylate[1, 2]. The sequential action of hydroxylating the glycyl α-carbon and then cleaving the carbon-amide bond are dependent upon the peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidylglycine amidoglycolate lyase (PAL) domains, respectively (Figure 5.1) [3-11].

PAM is responsible for activating peptide prohormones in vivo. Although a considerable body of mechanistic and structure/function data has been generated in an attempt to understand this redox chemistry [12-17], there are still crucial unanswered questions regarding electron transfer, dioxygen activation, and radical formation during C-H bond cleavage that preclude a comprehensive understanding of glycine hydroxylation. Crystallographers have stated that the reduction of PAM by ascorbate is collisional as they have been unable to identify a binding site for ascorbate. Mimosine, however, has been shown to be a competitive inhibitor against ascorbate suggesting that a binding site must exist [18].

This work attempts to elucidate possible binding sites for ascorbate using all-atom molecular dynamics simulations. Steered molecular dynamics (SMD) simulations are also employed in an attempt to confirm the computationally discovered binding sites of ascorbate by comparing the experimentally determined $K_M$ with the potential of mean force calculated using the Jarzynski relation. In SMD simulations, external forces are applied in a time-dependent manner to, for example, a ligand to facilitate unbinding from an active site. One can analyze the interactions of the unbinding ligand with the pocket, and the applied forces and ligand position as
a function of time yielding important structural information about the binding mechanism and structure-function relationships of the ligand-receptor complex [19]. By utilizing the Jarzynski relationship, one can relate the average potential of mean force derived from a series of SMD simulations to the average difference in free energy of two equilibrium states.

The Jarzynski relationship allows for the use of non-equilibrium calculations of equilibrium free energies. In 1997, Jarzynski proved that:

\[
\Delta G_{A \rightarrow B} = -\frac{1}{\beta} \ln \langle \exp (-\beta W_{A \rightarrow B}) \rangle = -\frac{1}{\beta} \ln \sum_{i=1}^{N} \frac{1}{N} \exp (-\beta W_{i,A \rightarrow B})
\]

where \(\Delta G\) is the Gibbs free energy difference, \(\beta = 1/k_B T\), \(W_i\) is the out of equilibrium work done onto the system when going from state A to state B, and the exponential average is done over an equilibrium ensemble only for state A. The only requirements for this equality to work are that the initial ensemble over state A be equilibrated, and that the exponential average be converged. There is, however, no requirement as to how the switch from state A to state B should be done (Figure 5.2).

Computationally, this is the power of the Jarzynski relationship as there are no requirements for how slowly one must switch from state A to state B. The real advantage of employing the Jarzynski relationship is that one only needs to equilibrate the initial state. This eliminates the necessity for a slow, quasi-static transformation from state A to state B. In an era of massively parallel computers available for short spans of time, this type of simulation is ideal where a complete Jarzynski style computation can be submitted in parallel and all data collected in the time of one simulated pulling. When small counts of CPUs are available for long periods of time, other methods may be preferred.
Methods

*Equilibrium Molecular Dynamics*

The PHM crystal structure (1SDW) was first obtained from the Protein Databank, and all catalytically superfluous molecules and ions were removed. The force field parameters for both Cu\(^{2+}\) domains were determined by normal mode analysis in the harmonic approximation. The Hessian was calculated for each methyl-capped Cu-containing fragment (Figure 5.3) separately using Density Functional Theory to obtain the bonding and bending force constants. DFT calculations were performed with the B3LYP hybrid exchange-correlation functional and the 6-31G* basis set. The equilibrium bond distances were found via geometry optimization. The partial charges of the coppers were the formal charges expected to exist prior to reduction by ascorbate. All other residues used potential parameters from the CHARMM27 force field.

The PHM system was then solvated in a 120 Å\(^3\) water box using the TIP3P water model to which Na\(^+\) and Cl\(^-\) counter-ions were added to a concentration of 100 mM. NAMD was then used to equilibrate the system. All PHM non-backbone atoms were fixed and the system was energy minimized for 10,000 steps followed by a minimization of all atoms for 10,000 steps. The system was then heated with C\(_\alpha\) constrained for 10 ps in the NVT ensemble (T=310.15 K), volume equilibrated in the NPT ensemble with C\(_\alpha\) constrained, and free dynamics performed in the NPT ensemble (P=1 atm, T=310.15 K) for 2.4 ns (Figure 5.4). Two separate systems were then created using the equilibrated PHM structure to probe for both ascorbate (ASC) and mimosine (MIM) binding sites. The desired probe (ASC or MIM) was added to the solution surrounding the equilibrated PHM structure to a concentration of ~10 mM. A new set of counter-ions was added to a concentration of 100 mM.
All PHM atoms were fixed and the system was energy minimized for 3,000 steps and dynamics run for 200 ps on the water and counter-ions. The ASC or MIM molecules were then released and, with PHM still fixed, the system was minimized for 5,000 steps and dynamics was performed for 500 ps. These steps were performed in the NVT ensemble (T=310.15 K). All atoms were then released from constraints, the system minimized for 3,000 steps, and free dynamics in the NPT ensemble (P=1 atm, T=310.15 K) run for 80 ns. All simulations described utilized periodic boundary conditions and the Particle Mesh Ewald (PME) method to calculate electrostatic forces without cutoff[20]. Van der Waals interactions were calculated with a cutoff of 12 Å and a switching distance of 10 Å. Bonded forces, short range non-bonded forces (within the cutoff), and long range electrostatics (outside the cutoff) were evaluated every femtosecond during the initial equilibration of the PHM structure. For the binding simulation, a multiple time-stepping algorithm[21, 22] was utilized with a 1 fs step for bonded force evaluation, 2 fs for short range non-bonded forces (within the cutoff), and 4 fs for long range electrostatics (outside the cutoff). Langevin dynamics was used to control the temperature using a damping coefficient of 5 ps$^{-1}$ with hydrogen atoms not coupled to the heat bath. Pressure was regulated via the hybrid Nose-Hoover[23] Langevin[24] piston method using a piston oscillation period of 100 fs and a damping time scale of 50 fs.

*Steered Molecular Dynamics*
Chapter Five: Computational Elucidation of Reductant Binding Sites in PAM

Upon binding of ascorbate or mimosine in their respective simulations, ten starting structures from each simulation were collected for multiple steering molecular dynamics simulations. External steering forces were then applied to pull the ligand, ASC or MIM, out of its binding site. The ligands were pulled under constant velocity in directions predetermined through visualization, one pull per each of the 20 collected starting structures (Figure 5.5).

Bonded forces, short range non-bonded forces (within the cutoff), and long range electrostatics (outside the cutoff) were evaluated every femtosecond for all steered molecular dynamics simulations. The pulling velocity was set at 0.00005 Å per time step (1 fs). A spring constant of 7 kcal/mol/Å² was used to constrain the ligand. This constraint is large enough to allow for the use of the stiff spring approximation[25, 26]. For all pulling simulations, the pulling force was applied to the atoms indicated in Figure 5.6. The force was only applied along the pulling direction during the simulation. The ligands were free from constraint in the plane perpendicular to the pulling vector. Trajectories were saved every 0.1 ps, and steering forces recorded every 10 fs.

Analysis of Trajectories

The methods used to construct the potential of mean force (PMF) from the steered molecular dynamics simulations were all based on Jarzynski’s equality and were independent of the friction coefficient [27]. The reaction coordinate was the increasing distance between the ligand and the binding site during the pulling simulation. Because drifting of the protein would change the distance between the ligand and the binding site throughout the simulation, several atoms on the side opposite of the binding site were fixed on the protein (Figure 5.7). Specifically, these were the Ca of LYS336 and ASN337. The use of 10 trajectories for both
ASC and MIM are sufficient as fewer slower pulling trajectories have been demonstrated to yield more accurate PMF values\[25, 26\]. The 50 Å/ns pulling velocity is sufficiently slow to produce accurate PMF values from the number of trajectories used here.

The pulling force was calculated using the following equation:

\[ F(t) = k (v t - (\vec{r}(t) - \vec{r}_0) \cdot \vec{n}) \]  

where \( F \) is the pulling force, \( t \) is time, \( k \) is the spring constant of the constraint, \( v \) is the pulling velocity, \( n \) is the pulling direction normal, and \( r(t) \) and \( r_0 \) are the positions of the ligand at time \( t \) and \( t_0 \).

Work was calculated by integrating the force over the distance pulled from the pulling trajectories:

\[ W(r) = \int_0^r F(r')dr' \]  

The PMF, or free-energy difference, was derived from work \( W \) utilizing Jarzynski’s equality as follows\[26, 28\]:

\[ \Delta G = -RT \ln \langle \exp \left( -\frac{W}{RT} \right) \rangle \]

where \( R \) is the universal gas constant and \( T \) is the absolute temperature. Previous work by the Schulten group has demonstrated that work distribution from Langevin dynamics satisfies a Gaussian distribution and can be expressed as a second-order cumulant expansion\[26\]:

\[ \Delta G = \langle W \rangle - \frac{\sigma W^2}{2kT} \]
where $\langle W \rangle$ is the average work from all trajectories and $\sigma_W$ is the standard deviation of the work distribution.

**Results**

*Ascorbate binding simulations*

The simulation for the ascorbate binding experiment reveals the probe diffusing though the aqueous solution around PHM. Some of the ligands search the surface of PHM and again diffuse. Some ascorbate molecules in the simulation search the surface and bind to it temporarily. One ascorbate molecule in the simulation, however, binds and remains bound for 15 ns. The criteria for determining the legitimacy of a binding site included two important guidelines. The binding site must be a depressed, hydrophobic pocket and not simply hydrogen bonding interactions on the surface of PHM. The binding should last on the order of $>10$ ns to ensure sufficient interaction strength. The ascorbate simulation was carried out for 79 ns and revealed only one potential binding site for ascorbate (Figure 5.8).

The binding site for ascorbate is a depression on the PHM surface that includes residues important for strong hydrogen bonding. These residues include ASP61, GLN198, and LYS152. A frame from the trajectory during the “ascorbate on” portion of the simulation is rendered below (Figure 5.9).

As a means of measuring the amount of time the particular binding ascorbate ligand spent bound to the enzyme, hydrogen bonding was monitored between the ascorbate ligand and the surface of PHM. The data indicates that ascorbate bound to PHM for $\sim 15$ ns before diffusing. This meets our criteria for legitimate binding and is depicted in Figure 5.10.

*Mimosine binding simulations*
The simulation for the mimosine binding experiment was performed in the same manner as that of the ascorbate binding experiment. Mimosine diffused freely throughout the solvent during the simulation. The mimosine simulation was carried out for ~82 ns, and only one potential binding site for mimosine was identified (Figure 5.11).

The binding site for mimosine is a depression on the PHM surface much like that of ascorbate and includes residues important for strong hydrogen bonding. The binding site residues include ASN45, ARG67, ARG100, and LYS98. A frame from the trajectory during the mimosine bound portion of the simulation is rendered in Figure 5.12.

In order to determine the amount of time mimosine was bound to PHM during the simulations, the hydrogen bonding between the bound ligand and the binding site was investigated. Figure 5.13 illustrates that mimosine seemed to bind longer than ascorbate perhaps suggesting a stronger interaction which would be consistent with what has been observed experimentally.

Because ascorbate and mimosine were shown to bind in close proximity but different binding pockets, the particle mesh Ewald electrostatic potential of PHM was calculated for the “on” and “off” states of both ligands. This illustrates that the electrostatic effects of mimosine binding would indeed affect the binding pocket of ascorbate. Thus, the seemingly competitive experimental data may not rule out slightly differing binding sites within a few angstroms of one another (Figures 5.14 & 5.15).

Steered Molecular Dynamics

The SMD simulations for both ascorbate and mimosine yielded force data that fluctuate between positive and negative values (Figure 5.16). This indicates that the thermal fluctuation of the ligand (ASC or MIM) is larger than the perturbation from the pulling. This shows that the
process by which the ligand unbinds is near equilibrium. No conformational changes were seen during the unbinding process with mild peaks in the force graph relating only to the breaking of hydrogen bonds.

The unbinding of the two ligands, ascorbate and mimosine, are nonequilibrium processes. The external work was sampled from repeated trajectories in order to calculate the potential of mean force using the Jarzynski equality (Figure 5.17). Jarzynski’s equality cannot be used directly unless the deviation of work distribution $\sigma_w^2 \leq 4kT$ which is generally not true for biological systems of the size simulated here[19]. Because standard deviation will grow as the ligand is pulled further from the binding site, the free energy estimated from the Jarzynski equality is dominated by smaller values of work. The cumulant expansion method was developed to solve this problem[25, 26] (Figure 5.18). Utilizing the cumulant expansion method improves the statistics of work distribution caused by having such limited sampling when calculating the PMF.

Discussion

The simulations performed in an attempt to elucidate the binding site for ascorbate, the reductant of PAM, have led to the discovery of a possible binding site. The successful elucidation of a binding site will help eliminate some of the proposed mechanisms and help determine where electron transfer occurs prior to the reduction of CuM. The mimosine simulation was used as a control in that an ascorbate binding site would either accommodate mimosine or a mimosine binding site would be very nearby. This proved to be the case in the proposed ascorbate binding site. Mimosine binds within a few angstroms of ascorbate.

Another method used as a way of verifying the proposed binding site was the calculation of the free energy of binding. While the calculated free energy for mimosine can be directly
compared with the experimental $K_I$ value (4 µM) as no chemistry is occurring, that of ascorbate is a $K_M$ (223 µM) and cannot be compared directly but is a good estimate. Using the equations for Gibbs free energy:

$$\Delta G = RT\ln(K)$$

where $K$ is the $K_d$ or $K_M$ for mimosine and ascorbate, respectively, calculated from the PMF values for free energy simulated. The calculated PMF values for ascorbate yields a theoretical $K_M$ of 200 µM. This is sufficiently close to justify consideration of the proposed binding site as valid. The theoretical $K_I$ of mimosine from the simulation results in a value of 1 µM which is also consistent with the experimental data within the accepted error for this type of free energy calculation.

While initial results are convincing, more work is needed to experimentally verify these initial findings. Mutants targeting the proposed binding site should be made and the effects of these mutations tested on reduction of PAM by ascorbate. A quicker method may be to perform in silico docking of a library of commercially available compounds. Hits for binding in the proposed binding site should be tested as competitive inhibitors against the reduction of PAM by ascorbate.
References


Chapter Five: Computational Elucidation of Reductant Binding Sites in PAM


Chapter Five: Computational Elucidation of Reductant Binding Sites in PAM


Figure 5.1. Peptidylglycine α-amidating monooxygenase reaction. Note, Asc represents ascorbate, deAsc represents semidehydroascorbate, PAM is peptidylglycine α-amidating monooxygenase, PHM is peptidylglycine α-hydroxylating monooxygenase, and PAL is peptidylglycine amidoglycolate lyase.
Figure 5.2. States A and B in which state A is the starting structure and state B is the final state post SMD simulation. Water and ions omitted for clarity.
Figure 5.3. Methyl-capped Cu_H and Cu_M domains in the PHM crystal structure prior to geometry optimization.
Figure 5.4. PHM crystal structure “soaked” with ascorbate. Note, water and ions not shown for clarity.
Figure 5.5. Illustration of the PHM secondary structure with bound ascorbate and the spring as the force vector applied during the steering molecular dynamics simulations.
Figure 5.6. Ascorbate and mimosine used to probe for a reductant binding site on PHM and the carbon atoms in which the force was applied during the steered molecular dynamics simulation.
Figure 5.7. PHM secondary structure with bound ascorbate and the fixed L336 and N337.
Figure 5.8. Ascorbate bound to the surface of PHM.
Figure 5.9. Ascorbate in the proposed binding pocket with hydrogen bonding illustrated as blue or red dashed lines.
Figure 5.10. Hydrogen bonding between ascorbate and the proposed PHM binding site.
Figure 5.11. Mimosine bound to the surface of PHM.
Figure 5.12. Mimosine in the proposed binding pocket with hydrogen bonding illustrated as blue dashed lines.
Figure 5.13. Hydrogen bonding between mimosine and the proposed PHM binding site.
Figure 5.14. PME electrostatic potential of PHM with “off” and “on” ascorbate.
Figure 5.15. PME electrostatic potential of PHM with “off” and “on” mimosine.
Figure 5.16. Force vs extension for both ascorbate and mimosine over a 5 Å reaction coordinate.
Figure 5.17. Work vs extension for ascorbate and mimosine unbinding.
Figure 5.18. Potential of mean force vs extension for ascorbate and mimosine unbinding.
Appending A

PCGAMESS Geometry Optimization Settings

File 1. \( L_3\text{Cu(I)}\)-thiophenolate geometry optimization (truncated)

! Geometry optimize the CuM cluster with thiophenolate anion coordinated
! (froze the carbons of the methyl caps to simulated the protein backbone)
! lanl2dz basis set
!
! Jon Belof/Edward W. Lowe
! Department of Chemistry
! University of South Florida
!
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4 ----- p-d potential ----- 
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File 2. L_3Cu(I)-H_2O geometry optimization (truncated)

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! Edward W. Lowe Jr
! Merkler Research Group
! Department of Chemistry
! University of South Florida
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File 3. L₃Cu(I) geometry optimization (truncated)

! Geometry optimize the CuM cluster with no water coordinated
(froze the carbons of the methyl caps to simulated the protein backbone)
lanl2dz
Jon Belof/Edward W. Lowe
Department of Chemistry
University of South Florida

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   -72.554828 2 93.2801074
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4 ----- s-d potential ----- 
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   23.8351825 1 185.2419886
   473.8930488 2  73.1517847
  157.6345823  2 14.6884157
4 ----- p-d potential ----- 
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   6.4990936 1 130.8345665
  351.4605395 2  53.8683720
  85.5016036  2 14.0989469
S-ECP GEN 10 2
 5 ----- d potential ----- 
   -10.000000 1 532.6685222
  -85.3593846 2 108.1342248
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  -0.9899295  2  2.3712569
5 ----- s-d potential ----- 
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  10.6284036 1 100.8245833
  223.6360469  2 53.5858472
Appendices

93.6460845  2  15.3706332
28.7609065  2  3.1778402
6  ----- p-d potential  ----- 
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  6.0969842  1  93.2808973
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53.6569778  2  7.8120535
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File 4. $L_3Cu(II)$ geometry optimization of CuM for NAMD parameterization

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!Edward W. Lowe
!Merkler Research Group
!University of South Florida
!05/04/07
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File 5. $L_3$Cu(II) geometry optimization of CuH for NAMD parameterization

!Optimization of CuH for the ascorbate binding site probe using NAMD
Appendices

Edward W. Lowe
Merkler Research Group
University of South Florida
05/04/07

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File 7. Geometry optimization of mimosine for NAMD parameterization

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$PDC PTSEL=CONNOLLY CONSTR=CHARGE SEND
$FORCE PURIFY=.TRUE. PRTIFC=.TRUE. DECOMP=.TRUE. SEND
$DATA
mimosine toppar
C1
c 1 cc2
c 2 cc3 1 ccc3
n 3 nc4 2 ncc4 1 dih4
c 4 cn5 3 cnc5 2 dih5
c 5 cc6 4 ccn6 3 dih6
o 2 oc7 3 oce7 4 dih7
c 4 cn8 3 cnc8 2 dih8
c 8 cc9 4 ccn9 3 dih9
n 9 nc10 8 ncc10 4 dih10
o 1 oc11 2 oce11 3 dih11
c 9 cc12 8 ccc12 4 dih12
o 12 oc13 9 occ13 8 dih13
o 12 oc14 9 occ14 8 dih14
h 3 hc15 2 hcc15 1 dih15
h 5 hc16 4 hcn16 3 dih16
h 6 hc17 5 hcc17 4 dih17
h 7 ho18 2 hoc18 3 dih18
h 8 hc19 4 hcn19 3 dih19
h 8 hc20 4 hcn20 3 dih20
h 9 hc21 8 hcc21 4 dih21
h 10 hn22 9 hnc22 8 dih22
h 10 hn23 9 hnc23 8 dih23
cc2 1.409701
cc3 1.399166
ccc3 118.654
nc4 1.368604
ncc4 121.037
dih4 -0.201
cn5 1.367139
cnc5 120.411
dih5 0.620
cc6 1.395360
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<td>ccc12</td>
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### File 8. General input for all PBA geometry optimizations

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$CONTRL ICHARG=-1 MULT=1 RUNTYP=OPTIMIZE SCFTYP=RHF COORD=CART
$END

$CONTROL UNITS=ANGS MAXIT=200 $END

$DFT DFTTYP=B3LYP METHOD=GRID $END

$BASIS GBASIS=N31 NGAUSS=6 $END

$STATPT METHOD=QA NSTEP=100 OPTTOL=0.0001 HESS=CALC $END

$GUESS GUESS=HUCKEL $END

$SYSTEM MWORADS=200 $END

$P2P P2P=.T. DLB=.T. $END

$DATA

pba geopt

C1

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Appendices

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<th>Y-Coordinate</th>
<th>Z-Coordinate</th>
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</tbody>
</table>

$END

File 9. General input for PBA single point energy calculations

$CONTRL COORD=CART ICHARG=-1 MAXIT=200 MPLEVL=2
MULT=1 RUNTYP=ENERGY SCFTYP=RHF UNITS=ANGS
$END
$BASIS DIFFS=.true. GBASIS=N311 NDFUNC=2
NFFUNC=1 NGAUSS=6 NPFUNC=1 POLAR=HONDO7
$END
$SCF DAMP=.false. DEM=.false. DIIS=.false.
DIRSCF=.true. EXTRAP=.true. RSTRCT=.false.
SHIFT=.false. SOSCF=.true.
$END
$STATPT METHOD=QA NSTEP=1000 OPTTOL=0.001
$END
$P2P P2P=.T. DLB=.T. $END
$FORCE TEMP=0
$END
$GUESS
GUESS=HUCKEL
$END
$SYSTEM
MWORDS=100
$END
$DATA
high level spe for RSE
$END

**File 10. General input for PBA radical geometry optimizations (truncated)**

$CONTRL ICHARG=-1 MULT=2 RUNTYP=OPTIMIZE SCFTYP=UHF COORD=CART
$END
$CONTROL UNITS=ANGS MAXIT=200 $END
$DFT DFTTYP=B3LYP METHOD=GRID $END
$BASIS GBASIS=N31 NGAUSS=6 $END
$STATPT METHOD=QA NSTEP=1000 OPTTOL=0.0001 HESS=CALC $END
$GUESS GUESS=HUCKEL $END
$SYSTEM MWORDS=200 $END
$P2P P2P=.T. DLB=.T. $END
$DATA
pba radical geopt

**File 11. General input for PBA radical single point energy calculations (truncated)**
Appendices

$CONTROL RUNTYP=ENERGY COORD=CART ICHARG=-1 MULT=2 $END
$CONTROL MAXIT=200 MLEVEL=2 SCFTYP=UHF UNITS=ANGS $END
$BASIS GBASIS=N311 NDFUNC=2 NFFUNC=1 NGAUSS=6 NPFUNC=1 $END
$STATPT NSTEP=1000 $END
$GUESS GUESS=HUCKEL $END
$SYSTEM MWORDS=200 $END
$DATA
high level spe for RSE

File 12. General input for cinnamate analog geometry optimizations (truncated)

$CONTROL ICHARG=-1 MULT=1 RUNTYP=OPTIMIZE SCFTYP=RHF COORD=CART $END
$CONTROL UNITS=ANGS MAXIT=10000 $END
$SYSTEM KDIAG=0 TIMLIM=999999999 AOINTS=DIST $END
$SMP CSMTX=.T. $END
$P2P P2P=.T. DLB=.T. $END
$SCF DIRSCF=.T. FDIFF=.F. SOSCF=.F. DIIS=.T. ETHERSH=2.0 $END
$DFT DFTTYP=B3LYP METHOD=GRID $END
$BASIS GBASIS=N31 NGAUSS=6 $END
$STATPT METHOD=QA NSTEP=1000 OPTTOL=0.0001 HESS=CALC $END
$GUESS GUESS=HUCKEL $END
$SYSTEM MWORDS=200 $END
$P2P P2P=.T. DLB=.T. $END
$DATA

Appendix B

PCGAMESS Hessian Calculation Input Files

File 13. Hessian calculation for $L_3Cu(I) CuM$ for NAMD parameterization (truncated)

$CONTROL SCFTYP=RHF RUNTYP=HESSIAN ICHARG=+1 MULT=1 COORD=ZMT NZVAR=105 $END
$CONTROL MAXIT=100 ECP=SBK $END
$SYSTEM TIMLIM=9999999999 MWORDS=200 $END
$SCF DIRSCF=.TRUE. $END
Appendices

$STATPT NSTEP=1000 HESS=CALC SEND
$GUESS GUESS=HUCKEL SEND
$BASIS GBASIS=SBK SEND
$ELPOT IEPOT=1 WHERE=PDC SEND
$PDC PTSEL=CONNOLLY CONSTR=CHARGE SEND
$FORCE PURIFY=.TRUE. PRTIFC=.TRUE. DECOMP=.TRUE. SEND
$ZMAT IZMAT(1)=1,2,1,1,3,2,1,4,2,1,5,1,1,6,5,1,7,5,1,8,2,1,9,4,1,10,9,
1,11,8,1,12,11,1,13,8,1,14,13,1,15,14,1,16,14,1,17,13,1,18,17,1,19,17,
1,20,14,1,21,16,1,22,21,1,23,20,1,24,23,1,25,24,1,26,25,1,27,26,1,28,26,
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13,
$END
$DATA


$CONTRL ScFTYP=RHF RUNTYP=HESSIAN ICHARG=+2 MULT=2 COORD=ZMT
NZVAR=105 $END
$CONTRL MAXIT=100 ECP=SBK $END
$SYSTEM TIMLIM=9999999999 MWORDS=200 $END
$SCF DIRSCF=.TRUE. $END
$STATPT NSTEP=1000 HESS=CALC SEND
$GUESS GUESS=HUCKEL SEND
$BASIS GBASIS=SBK SEND
$ELPOT IEPOT=1 WHERE=PDC SEND
$PDC PTSEL=CONNOLLY CONSTR=CHARGE SEND
$FORCE PURIFY=.TRUE. PRTIFC=.TRUE. DECOMP=.TRUE. $END
$ZMAT IZMAT(1)=1,2,1,1,3,2,1,4,2,1,5,1,1,6,5,1,7,5,1,8,2,1,9,4,1,10,9,
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1,20,14,1,21,16,1,22,21,1,23,20,1,24,23,1,25,24,1,26,25,1,27,26,1,28,26,
$CONTRL SCFTYP=RHF RUNTYP=HESSIAN ICHARG=0 MULT=1 COORD=ZMT
NZVAR=54 $END
$CONTRL MAXIT=75 $END
$SYSTEM TIMLIM=999999999 MWORDS=235 $END
$SCF DIRSCF=.TRUE. $END
$STATPT NSTEP=1000 $END
$BASIS GBASIS=N31 NGAUSS=6 $END
$ELPOT IEPOT=1 WHERE=PDC $END
$PDC PTSEL=CONNOLLY CONSTR=CHARGE $END
$FORCE PURIFY=.TRUE. PRTIFC=.TRUE. DECOMP=.TRUE. $END
$ZMAT IZMAT(1)=1,2,1,1,3,2,1,4,3,1,5,4,1,6,2,1,7,6,1,8,6,1,9,5,1,10,1,
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17,12,11,2,18,8,7,2,19,10,1,2,20,7,5,3,4,3,2,1,3,5,4,3,2,3,6,2,3,4,3,7,4,3,2,3,8,7,4,3,3,9,5,4,3,10
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3,19,10,1,2,3,20,9,5,4,
$END
$ZMAT DLC=.TRUE. AUTO=.TRUE. $END
$DATA

ascorbate toppar

File 15. Hessian Calculation for ascorbic acid for NAMD parameterization (truncated)

$CONTRL SCFTYP=RHF RUNTYP=HESSIAN ICHARG=0 MULT=1 COORD=ZMT
NZVAR=54 $END
$CONTRL MAXIT=75 $END
$SYSTEM TIMLIM=999999999 MWORDS=235 $END
$SCF DIRSCF=.TRUE. $END
$STATPT NSTEP=1000 $END
$BASIS GBASIS=N31 NGAUSS=6 $END
$ELPOT IEPOT=1 WHERE=PDC $END
$PDC PTSEL=CONNOLLY CONSTR=CHARGE $END
$FORCE PURIFY=.TRUE. PRTIFC=.TRUE. DECOMP=.TRUE. $END
$ZMAT IZMAT(1)=1,2,1,1,3,2,1,4,3,1,5,4,1,6,2,1,7,6,1,8,6,1,9,5,1,10,1,
1,11,7,1,12,1,1,13,4,1,14,1,15,1,17,1,18,1,19,10,1,20,9,1,21,2,1,2,4,3,1,2,2,5,4,3,
2,6,2,3,2,7,4,3,2,8,7,4,2,9,5,4,2,10,1,2,2,1,1,7,4,2,12,11,7,2,13,4,3,2,14,7,4,2,15,117,2,16,11,7,2,
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3,19,10,1,2,3,20,9,5,4,
$END
$ZMAT DLC=.TRUE. AUTO=.TRUE. $END
$DATA

ascorbate toppar

File 16. Hessian Calculation for mimosine for NAMD parameterization (truncated)
Appendix C

NAMD Configuration Files

File 17. General configuration file for the rapid equilibration of proteins

# Equilibration run
#
# Monday May 26th, 2008
#
# Edward W. Lowe Jr
# Merkler Research Group
# Department of Chemistry
# University of South Florida

structure       dbm_for_equil.solvated.ionized.psf
coordinates    dbm_for_equil.solvated.ionized.pdb
seed 238897325
binaryoutput   on
outputname  dbm_equilibration
DCDfile  dbm_equilibration.dcd

# output frequency
outputenergies  1000
outputtiming  1000
restartfreq  10000
DCDfreq  1000

# integration parameters
timestep  1.0
rigidBonds  all
nonbondedFreq  1
fullElectFrequency  1
stepspercycle  10

# FF
paraTypeCharmm  on
parameters  par_all22_prot+Cu+ASC+MIM.inp
parameters  par_all27_prot_lipid_na.inp
exclude  scaled1-4
1-4scaling  1.0
switching  on
cutoff  12.0
switchdist  10.0
pairlistdist  14.0

# PBC
cellBasisVector1  94.0  0.0  0.0
cellBasisVector2  0.0  94.0  0.0
cellBasisVector3  0.0  0.0  94.0
cellOrigin  0.0  0.0  0.0
wrapall  on

# PME
PME  on
PMEGridSizeX  96
PMEGridSizeY  96
PMEGridSizeZ  96
# set temperature to 0 initially
temperature   0

# fix the backbone atoms and coppers in DBM
fixedAtoms    on
fixedAtomsForces on
fixedAtomsFile dbm_for_equil.solvated.ionized.fixed.pdb
fixedAtomsCol  B

# turn on constraints for all of the alpha carbons in DBM
constraints    on
consRef       dbm_for_equil.solvated.ionized.constrained.pdb
consKFile     dbm_for_equil.solvated.ionized.constrained.pdb
consKCol      B

# set piston will large damping
langevin      on
langevinDamping  5
langevinTemp   310.15
langevinHydrogen off
langevinPiston on
langevinPistonTarget 1.01325
langevinPistonPeriod 100
langevinPistonDecay 50
langevinPistonTemp 310.15
useGroupPressure yes

# run one step to get into scripting mode
minimize     0

# turn off langevin pressure control dynamics
langevinPiston off

# minimize non-backbone atoms
minimize     10000

# minimize all atoms
fixedAtoms   off
minimize 10000

# heat with alpha carbons constrained
run 10000

# equilibrate volume with alpha carbons constrained (NPT on)
langevinPiston on
run 10000

# equilibrate volume without constraints (1ns NPT)
constraintScaling 0
run 1000000

File 18. General configuration file for long molecular dynamics simulations using multiple stepping algorithm

#
# PHM domain with bound coppers
# NPT 80 ns run for asc binding site
# Saturday July 20th, 2007
#
# Edward W. Lowe Jr
# Merkler Research Group
# Department of Chemistry
# University of South Florida

structure PHM+ox_asc_solvated_ionized.psf
coordinates PHM+ox_asc_solvated_ionized.pdb
bincoordinates PHM+ox_asc_NPT_WRAPALL_17.restart.coor
extendedsystem PHM+ox_asc_NPT_WRAPALL_17.restart.xsc
binvelocities PHM+ox_asc_NPT_WRAPALL_17.restart.vel

seed 238897325

binaryoutput on
outputname PHM+ox_asc_NPT_WRAPALL_18
dcdfile PHM+ox_asc_NPT_WRAPALL_18.dcd
# output frequency
outputenergies 10000
outputtiming 10000
restartfreq 10000
DCDfreq 10000

# integration parameters
timestep 1.0
rigidBonds all
nonbondedFreq 2
fullElectFrequency 4
stepspercycle 20

# FF
paraTypeCharmm on
parameters par_all22_prot+Cu+ASC.inp
parameters par_all27_prot_lipid_na.inp
exclude scaled1-4
1-4scaling 1.0
switching on
cutoff 12.0
switchdist 10.0
pairlistdist 14.0

# PBC
cellBasisVector1 120.0 0.0 0.0
cellBasisVector2 0.0 120.0 0.0
cellBasisVector3 0.0 0.0 120.0
cellOrigin 0.0 0.0 0.0
wrapall on

# PME
PME on
PMEGridSizeX 122
PMEGridSizeY 122
PMEGridSizeZ 122

# set piston will large damping
langevin    on
langevinDamping   5
langevinTemp    310.15
langevinHydrogen off
langevinPiston    on
langevinPistonTarget 1.01325
langevinPistonPeriod   100
langevinPistonDecay  50
langevinPistonTemp 310.15
useGroupPressure yes

# run one step to get into scripting mode
minimize 0

# MD for 80 ns
run 80000000

File 19. General configuration file for steered molecular dynamics simulations

# Edward W. Lowe Jr
# Merkler Research Group
# Department of Chemistry
# University of South Florida
#
# Constant Velocity Pulling of reductant from PHM

structure          PHM+ox_asc1_bound_solvated_ionized.psf
coordinates         PHM+ox_asc1_bound_solvated_ionized.pdb
bincoordinates   1.coor
outputName          asc_pulling_1_022408
set temperature     310
firsttimestep       0

# Input
paraTypeCharmm    on
parameters         par_all22_prot+Cu+ASC+MIM7.inp
parameters   par_all27_prot_lipid_na.inp
temperature          $temperature
wrapWater on
wrapAll               on

# Force-Field Parameters
exclude              scaled1-4
1-4scaling           1.0
cutoff               12.
switching            on
switchdist           10.
pairlistdist         14.

# Integrator Parameters
timestep             1.0 ;# 1fs/step
nonbondedFreq        1
fullElectFrequency   1
stepspercycle        10

# Constant Temperature Control
langevin             off
langevinDamping      5
langevinTemp         $temperature
langevinHydrogen     no
restartfreq          500
dcdfreq              500
xstFreq              500
outputEnergies       100
outputPressure       100

# Fixed Atoms Constraint
if {1} {  
fixedAtoms           on
fixedAtomsFile       PHM+ox_asc1.SMD.ref
fixedAtomsCol        B
}

SMD                    on
SMDFile                PHM+ox_asc1.SMD.ref
SMDk                   7
SMDVel                 0.0001
SMDDir -0.467180057326 -0.0811620777483 0.880429163064
SMDOutputFreq 10

run 1000000
Appendices

Appendix D

Monooxygenase X: Homology modeling results

List of Figures

(Figure)

Figure A.1. Monooxygenase X solvated and ionized prior to equilibration using NAMD.
Figure A.2. Monooxygenase X in ribbon representation of the secondary structure after equilibration with NAMD.
Figure A.3. The rmsd versus time graph for the equilibration of Monooxygenase X using NAMD. Rmsd = 1.86.
Appendices

Appendix E

*Equilibration results of oxidized PHM*

(Figure)

*Figure A.4. PHM crystal structure in ribbon format solvated prior to equilibration with NAMD.*
Figure A.5. The time versus rmsd for the equilibration of oxidized PHM with NAMD. Rmsd = 2.057
Appendix F

Dopamine β-Monooxygenase: Homology model

(Figure )

Figure A.6. Dopamine β-monooxygenase homology model prior to refinement with NAMD.
Figure A.7. The rmsd versus time for the equilibration of dopamine β-monooxygenase homology model. Rmsd = 1.86
Appendices

Appendix G

Synthesis of $^{14}$C-mimosine

(Figure )

Figure A.8. Mimosine synthase reaction scheme.
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About the Author

Edward W. Lowe, Jr. (Will) graduated as valedictorian at A. Crawford Mosley High School in Panama City, FL in 2000. Will relocated to Tampa, FL in the Fall of 2000 to attend the University of South Florida. He graduated with Honors receiving a bachelors degree in chemistry in 2003. Will entered the chemistry graduate program at USF in 2003 and received his Ph.D. in the Fall 2008. When not performing geometry optimizations or molecular dynamics simulations in his spare time, Will enjoys power lifting having won the “Strongest Bull” competition in 2007. Above all, Will enjoys spending time with his wife, Kim, and daughter Addison.