

Peptidylglycine α -amidating monooxygenase as a therapeutic target or biomarker

David Merkler¹, Aidan Hawley¹, Betty Eipper², and Richard Mains²

¹University of South Florida

²UConn Health Center

July 28, 2021

Abstract

Peptides play a key role in controlling many physiological and neurobiological pathways. Many bioactive peptides require a C-terminal α -amide for full activity. The bifunctional enzyme catalyzing α -amidation, peptidylglycine α -amidating monooxygenase (PAM), is the sole enzyme responsible for amidated peptide biosynthesis, from *Chlamydomonas reinhardtii* to *Homo sapiens*. Many neuronal and endocrine functions are dependent upon amidated peptides; additional amidated peptides are growth promoters in tumors. The amidation reaction occurs in two steps, glycine α -hydroxylation followed by dealkylation to generate the α -amide product. Currently, most potentially useful inhibitors target the first reaction, which is rate-limiting. PAM is a membrane-bound enzyme that visits the cell surface during peptide secretion. PAM is then used again in the biosynthetic pathway, meaning that cell-impermeable inhibitors or inactivators could have therapeutic value for the treatment of cancer or psychiatric abnormalities. To date, inhibitor design has not fully exploited the structures and mechanistic details of PAM.

1. Introduction

Bioactive peptides play a key role in controlling many complex pathways including blood glucose (insulin and glucagon, for example), salt and water balance (vasopressin, atrial natriuretic peptide), appetite (cholecystokinin, α -melanocyte stimulating hormone, leptin), reproduction (gonadotropin hormone releasing hormone, follicle stimulating hormone) and gastrointestinal function (gastrin, motilin). Each of these peptides is synthesized from a newly synthesized precursor protein as it moves from its site of synthesis in the endoplasmic reticulum, through the secretory pathway lumen and into the secretory granules from which it is released in response to the appropriate combination of stimuli (Chrétien & Mbikay, 2016; Clark & Lowry, 2016; Kumar, Mains, & Eipper, 2016). A limited set of subtilisin-like endoproteases, carboxypeptidase B-like exoproteases and other post-translational processing enzymes convert these precursors, which are often inactive, into active products as they move through the secretory pathway (**Fig.1A**). The activity of many bioactive peptides requires α -amidation of their C-terminal residue; lacking an amidated C-terminus, their ability to bind to their receptors (generally a G Protein Coupled Receptor) is greatly reduced. Consistent with this, Peptidylglycine α -Amidating Monooxygenase (PAM), the only enzyme known to catalyze the formation of α -amidated peptides, is an essential enzyme. Mice lacking PAM survive only until mid-gestation (Czyzyk et al., 2005) and neither flies nor zebrafish lacking PAM are viable (Kolhekar, Roberts, et al., 1997; Kumar et al., 2018). Mice having a single functional *Pam* gene exhibit a wide variety of deficits, ranging from altered inhibitory synaptic neurotransmission to increased anxiety-like behavior and an inability to maintain body temperature in a cold environment (Bousquet-Moore et al., 2010; Gaier, Eipper, & Mains, 2014; Gaier et al., 2013). Genetic studies have identified *PAM* as a risk factor for type 2 diabetes (Steinhorsdottir et al., 2014; Thomsen et al., 2018).

PAM is a bifunctional enzyme: its monooxygenase domain (peptidylglycine α -hydroxylating monooxygenase, PHM: EC 1.14.17.3) catalyzes the copper- and ascorbate-dependent α -hydroxylation of its peptidylglycine

substrates; its lyase domain (peptidyl- α -hydroxyglycine α -amidating lyase, PAL: EC 4.3.2.5; also called peptidylamidoglycolate lyase) then generates the α -amidated product peptide plus glyoxylate (**Fig.1B**). C-terminal amidation can alter peptide structure, confer resistance to proteolytic degradation and reduce the effects of pH on receptor binding (Kahns & Bundgaard, 1991; Luxmi, Mains, King, & Eipper, 2021). Extensive mechanistic studies and structural studies facilitated the development of PAM inhibitors. In this brief review we will summarize several successful approaches taken to develop inhibitors of PHM and discuss observations indicating that PAM may serve as a useful therapeutic target or biomarker.

2. Πεπτιδε α -Αμιδατιον ιν Τωο Στεπς

2A. The PHM Reaction: Progress in studies on the formation of α -amidated peptides depended crucially on three key findings. First, studies conducted from the 1950's to the early 1980's made it clear that the proprotein precursors to peptide-amides always had a Gly residue located to the C-terminal side of the residue that was to be α -amidated (reviewed in (Kumar, Mains, et al., 2016)) (**Fig.1A** and **B**). Second, development of the first useable enzymatic assay of peptide amidation by Bradbury, Finnie and Smyth (A.F. Bradbury, Finnie, & Smyth, 1982). Third, the realization that pituitary cells maintained in serum-free culture medium efficiently stored and secreted POMC products, but were unable to produce α -amidated peptides (Eipper, Glembotski, & Mains, 1983). Taken together, these observations lead to the conclusion that the reaction depended on copper ions, reduced ascorbic acid and oxygen, followed by the purification of PHM, PAL and PAM from a variety of sources (Eipper et al., 1991; Murthy, Keutmann, & Eipper, 1987; Murthy, Mains, & Eipper, 1986; P. P. Tamburini, Young, Jones, Palmesino, & Consalvo, 1990) and the cloning and expression of PAM cDNAs from several species (Bertelsen et al., 1990; Eipper et al., 1987; Glauder, Ragg, Rauch, & Engels, 1990; Mizuno et al., 1987; Ouafik et al., 1992; Stoffers, Green, & Eipper, 1989; Stoffers, Ouafik, & Eipper, 1991). Although the lyase reaction catalyzed by PAL can occur at elevated pH in the absence of enzyme, phylogenetic studies support the presence of a bifunctional, integral membrane PAM protein (**Fig.1C**) in the last eukaryotic common ancestor (Bäck, Mains, & Eipper, 2021; Kumar, Mains, et al., 2016).

Most of the enzymological and all of the crystallographic studies of PHM and PAL have been carried out using the soluble, protease-resistant catalytic cores of PHM (PHMcc) and PAL (PALcc) (**Fig.1C**). Endoproteolytic digestion of lysates prepared from mammalian cells expressing exogenous PAM and of tissues that express high levels of endogenous PAM revealed that both catalytic activities were remarkably resistant to proteolytic degradation (Husten & Eipper, 1994; Husten, Tausk, Keutmann, & Eipper, 1993). For rat PAM, PHMcc consists of residues 42–356 (Eipper, Quon, Mains, Boswell, & Blackburn, 1995; Kolhekar, Keutmann, Mains, Quon, & Eipper, 1997) and PALcc consists of residues 498–820 (Kolhekar et al., 2002; Kolhekar, Quon, Berard, Mains, & Eipper, 1998). The crystal structures of recombinant PHMcc and PALcc have been extensively studied (Chufan, De, Eipper, Mains, & Amzel, 2009; Prigge, Eipper, Mains, & Amzel, 2004; Prigge, Kolhekar, Eipper, Mains, & Amzel, 1997; Siebert et al., 2005). PAM, PHM, and PAL purified from natural sources and soluble, recombinant PHM, PAL and bifunctional PAM have provided additional insight into the reaction mechanism (**Fig.1C**) (Bertelsen et al., 1990; Miller et al., 1992). To date, it has not been possible to obtain high resolution structural data for any bifunctional PAM proteins. Studies of the PHM and PAL components of bifunctional PAM are accomplished by employing the appropriate assays: O₂ consumption from a glycine-extended peptide is specific for PHM and glyoxylate production from a carbinolamide is specific for PAL. Although few studies have utilized human PAM, PHM or PAL, the highly conserved nature of the PHM and PAL active sites (Bäck et al., 2021; Kumar, Mains, et al., 2016) suggests that the catalytic properties of all of the various PAM, PHM and PAL proteins mentioned above are quite similar. Readers are encouraged to review the cited references if the exact source of the proteins is important to their research goals. Despite many attempts to express fully active PAM in bacterial, yeast, and insect systems, mammalian cell lines remain the system of choice. PHMcc has been expressed in *E. coli* with an N-terminal fusion to thioredoxin, but with a yield of only ~100 μ g/liter of *E. coli* (Handa, Spradling, Dempsey, & Merkler, 2012). Production of recombinant rat PHMcc, PALcc and rat MTC PAM in Chinese hamster ovary (CHO) cells has yielded >100 mg of pure enzyme (Bauman, Ralle, & Blackburn, 2007; Miller et al., 1992), sufficient enzyme to support spectroscopic studies (Blackburn, Rhames, Ralle, & Jaron, 2000; Eipper

et al., 1995; Evans, Blackburn, & Klinman, 2006; Jaron & Blackburn, 1999), structural biology (Chufan et al., 2009; Prigge et al., 1997; Prigge, Kolhekar, Eipper, Mains, & Amzel, 1999), and amidated peptide production at the multi-gram level (Ray et al., 1993).

PHM function requires two bound copper ions, Cu_H (with three His ligands) and Cu_M (with two His and one Met ligand) (Maheshwari et al., 2018; Prigge et al., 2004; Prigge et al., 1999; Siebert et al., 2005). Copper is easily lost from PHM, meaning that assaying PHM activity in crude lysates or in serum samples (Gaier et al., 2012) requires the addition of exogenous copper and analysis of purified PHM and PAM often requires replacement of copper lost during purification (Blackburn et al., 2000; Evans et al., 2006; Jaron & Blackburn, 1999; Murthy et al., 1986). The first step in the amidation reaction is the reduction of Cu(II)_H and Cu(II)_M to Cu(I) by ascorbate (**Fig. 2A**, Step 1). Studies show that the kinetic mechanism is equilibrium ordered, with the glycine-extended substrate binding first to reduced enzyme (**Fig. 2A**, Step 2), followed by the binding of O₂ to Cu(I)_M (**Fig. 2A**, Step 3). Formation of the PHM product, the (*S*)- α -hydroxyglycine derivative (Cowley, Tian, & Solomon, 2016; Ping, Mounier, & May, 1995), proceeds from the PHM-Cu(I)_H-Cu(I)_M-substrate-O₂ complex (**Fig. 2A**, Step 4) and regenerates oxidized PHM-Cu(II)_H-Cu(II)_M, preparing the enzyme for the next catalytic cycle (**Fig. 2A**, Step 5).

The chemical mechanism for PHM catalysis has been intensively debated (Chen, Bell, Eipper, & Solomon, 2004; Cowley et al., 2016; W.A. Francisco, Merkle, Blackburn, & Klinman, 1998; Jaron & Blackburn, 1999; Klinman, 2008; Kulathila, Merkle, & Merkle, 1999; Ping et al., 1995); it is agreed that a substrate-based radical on the α -carbon of the glycine is an intermediate and that a copper-superoxo species, Cu_M(II)-O₂^{*}, is responsible for hydrogen atom abstraction from the substrate glycine. Recent work from Wu et al. (Wu et al., 2019) suggests that an unusual (μ -O^{*})(μ -OH)Cu(II)_MCu(II)_H is the species responsible for hydrogen atom abstraction from substrate. In addition to the uncertainties about the reduced oxygen species responsible for hydrogen atom abstraction, there remain questions about the structure of the bifunctional PAM, binding site(s) for ascorbate, the electron transfer pathways between the two copper atoms and from the copper atoms to the substrate during catalysis, potential domain motion in PHM to reduce the 11Å distance between the Cu(II), and the possibility of carbinolamide channeling between the PHM and PAL active sites in PAM. Note that development of the current cohort of PHM inhibitors (discussed below) has progressed without definitive answers to these questions. Thus, the PHM and PAL inhibitors developed to date are substrate analogs. The rational design of second and third generation PHM inhibitors would benefit from a resolution of the mechanistic uncertainties, fostering the design of tight-binding transition-state analog inhibitors.

2B. The PAL Reaction: Like PHM, PAL is a metalloenzyme; PAL contains a bound Zn(II) and a bound Ca(II) (Bell et al., 1997; Chufan et al., 2009; De, Bell, Blackburn, Mains, & Eipper, 2006). PAL catalyzes the dealkylation of the (*S*)- α -hydroxyglycine derivative to the corresponding amide and glyoxylate (**Fig. 2A**, Step 5) (Katopodis, Ping, & May, 1990; Perkins, Husten, & Eipper, 1990). To date, evidence about channeling of the peptidyl- α -hydroxyglycine product from PHM to PAL is conflicting (W.A. Francisco et al., 1998; Moore & May, 1999). One major site for PHM and PAL catalysis is the acidic secretory granules with an internal pH ~ 5 (Mains & May, 1988). The α -hydroxyglycine derivative (a carbinolamide) is stable at this pH (Bundgaard & Kahns, 1991); thus, PAL is required for effective amide formation. The bound Ca(II) has a structural role in PAL, while the bound Zn(II) is likely involved in catalysis (Chufan et al., 2009; Takahashi et al., 2009). The precise role played by Zn(II) is not yet clear; suggestions include Zn(II)-bound water or hydroxide serving as a general base for proton abstraction from the hydroxyl group (Bell et al., 1997; Takahashi et al., 2009) and Zn(II)-coordination to the hydroxyl group to lower its pK_a, facilitating proton transfer to a tyrosyl phenolate in the active site (Chufan et al., 2009). Model studies by Tenn *et al.* (Tenn et al., 2007) suggest that carbinolamide dealkylation is not base-catalyzed and is independent of the pK_a of the hydroxyl group of the carbinolamide. Instead, these authors argue that carbinolamide dealkylation is dependent upon the nucleofugality of the departing amidate. If so, the PAL-bound Zn(II) could assist in the departure of the amidated product. Additional studies of PAL catalysis are required to address these mechanistic questions.

In the few cases examined, tissue lysates contained substantially more α -amidated peptide than peptidyl-

glycine precursor and levels of peptidyl- α -hydroxyglycine intermediate were well below peptidylglycine levels (Yin et al., 2011). These observations are consistent with the higher V_{\max} values reported for PAL vs PHM. As discussed below, the PAL domain plays an essential role in the ability of cells to retrieve membrane PAM from the cell surface. This suggests that inhibitors targeted to the active site of PAL might provide an effective means of inhibiting peptide amidation by reducing the amount of PAM available to amidate newly synthesized peptidylglycine substrates entering immature secretory granules. It is intriguing that the PAM loci most frequently associated with human diseases are in the PAL domain, not the PHM domain (Steinhorsdottir et al., 2014; Thomsen et al., 2018).

3. Effective PHM and PAL Inhibitors Were Developed .

For a period of about 25 years, from 1990 to 2015, there was considerable interest in developing PHM inhibitors and inactivators (Ali et al., 2015; Bolkenius, Ganzhorn, Chanal, & Danzin, 1997; F. Cao et al., 2011; A.G. Katopodis & S.W. May, 1990; Langella et al., 2010). The hope was that a PHM-targeted compound would be therapeutically useful in the treatment of human disease and/or would contribute to our understanding of PHM. Compounds that would intercept the radical intermediate were developed (Zabriskie, Cheng, & Vederas, 1992; Zabriskie, Klinge, Szymanski, Cheng, & Vederas, 1994) and PAM labeling with mechanism-based inactivators (suicide substrates) was used to try to identify the active site amino acids critical to catalysis. There was less interest in developing PAL-targeted compounds, the thought being that the non-enzymatic dealkylation of the accumulated carbinolamide intermediate would supply sufficient levels of the amidated product to alleviate PAL inhibition; as discussed above, PAL-targeted compounds that altered the trafficking of PAM could still prove useful in manipulating amidation *in vivo* .

Many drugs exhibit cell toxicity. Such therapeutics have greater toxicity towards diseased cells relative to healthy cells and must be used properly in treating a disease. In addition, ongoing research will lead to “molecular zip codes”, a protein or a small molecule that binds tightly and specifically to uniquely target diseased cells (Enbäck & Laakkonen, 2007). The attachment of a drug to a molecular zip code would enable the delivery of the drug specifically to the diseased cells – a strategy enabling the safe use of highly toxic drugs to treat human disease. A toxic PHM-specific inhibitor could be valuable therapeutically if appended to the appropriate molecular zip code. The production of amidated autocrine growth factors by tumor cells suggests that high-affinity cell-impermeant PHM inhibitors might prove useful in controlling the growth of these cells. Most neurons and endocrine cells cleave PHM from PAL within secretory granules, and release the PHM along with peptide. These cells would presumably ingest less of the toxic PHM-specific inhibitor than would cancerous cells, since tumor cells usually leave much of their PAM intact while making the amidated autocrine growth factors which enhance tumorigenicity.

Another use of biomedical significance for a PHM-targeted compound would be as a PAM-specific imaging agent. PHM is a biomarker for specific cancers (Jiménez et al., 2003; Rocchi et al., 2004; Scopsi et al., 1998; Thouënon et al., 2007), post-polio syndrome (Gonzalez et al., 2009), and neural dysfunction (Bousquet-Moore et al., 2010). In sum, the toxicity concern about a PHM inhibitor/inactivator is short-sighted and is limiting work to develop PHM-dependent imaging agents and provides few options when a PHM-specific molecular zip code becomes available.

3A. PHM Inhibitors: The inhibitory effects of divalent metal ion chelators on peptide amidation became apparent as soon as different buffers were tested for use in enzyme assays of tissue lysates (Eipper, Mains, & Glembotski, 1983). Inhibition by diethyldithiocarbamate (DDC) was reversed only by the addition of Cu(II), providing the first evidence that PAM/PHM catalysis was copper-dependent; no other divalent metal, including Zn(II), restored activity (Eipper, Mains, et al., 1983; Eipper, Park, Keutmann, & Mains, 1986). Other metal chelators like EDTA (Merkler, Kulathila, Young, Freeman, & Villafranca, 1993) and disulfiram inhibit PAM/PHM reversibly (Mains, Park, & Eipper, 1986). Disulfiram (Antabuse) is used for the treatment of alcohol abuse (Kranzler & Soyka, 2018) and has anti-cancer activity (Corsello et al., 2020). The possible link between the anti-cancer activity and PHM inhibition has not been directly investigated. Disulfiram has been used to inhibit α -amidated peptide production in cultured mammalian cells and in rats (Mains et al., 1986; Mueller & Altarac, 1995; Rondeel et al., 1995).

Most of the PHM inhibitors developed to date likely bind to the peptide substrate site within the PHM active site (Prigge et al., 1997). A review of the substrates oxidized by PHM is required to provide context for the PHM inhibitors. Only peptides with a C-terminal glycine are PHM substrates *in vivo* (K.A. Johnson, Paisley-Flango, Tangarone, Porter, & Rouse, 2007). Synthetic peptides with a C-terminal D-Ala are substrates leading to the corresponding α -amidated peptide and pyruvate (Landymore-Lim, Bradbury, & Smyth, 1983). However, the D-Ala-extended peptides are relatively poor substrates with low V_{\max}/K_m ratios. The V_{\max}/K_m ratio provides an estimate of the first-order rate constant for the conversion of substrate to product and is obtained using a substrate concentration far below its K_m value; when comparing different substrates, it provides a measure of enzyme specificity (K. A. Johnson, 2019). For example, the V_{\max}/K_m ratio for *N*-dansyl-Tyr-Val-D-Ala is 0.06 times the ratio for *N*-dansyl-Tyr-Val-Gly; the V_{\max}/K_m ratio for *N*-benzoyl-D-Ala is 0.0005 times the ratio for *N*-benzoylglycine (Merkler et al., 2008). Systematic studies to determine the effect of the penultimate amino acid on the kinetics of amidation showed that glycine-extended peptides with a penultimate aromatic, hydrophobic, or sulfur-containing amino acid had the highest V_{\max}/K_m ratios while those with a penultimate Lys or Arg had the lowest V_{\max}/K_m ratios (Merkler et al., 1993; P. P. Tamburini et al., 1990). α -Amidated peptides with a C-terminal amino acid amide for all 20 of the common amino acids are known (Eipper, Stoffers, & Mains, 1992). Studies aimed at producing amidated peptides in cell lines using endogenous or exogenous PAM indicate that the general rules for effective amidation developed by Tamburini et al. are not directly applicable to the conditions encountered as PAM and its substrates move through the secretory pathway in cells (Chew et al., 2005). A free carboxylate is required on the C-terminal glycine for amidation. The methyl or ethyl esters are not substrates, but are inhibitors of relatively low affinity (P.P. Tamburini et al., 1988). Similarly, the amidated products are low affinity inhibitors (Glembotski, Eipper, & Mains, 1984).

α -Amidated peptide hormones range considerably in length, from 3 amino acids for TRH to 52 amino acids for adrenomedullin. Much larger proteins that terminate with a C-terminal amide and generate α -amidated chemomodulatory peptides have been identified in the green algae, *Chlamydomonas reinhardtii* (Luxmi, Kumar, Mains, King, & Eipper, 2019). When expressed exogenously, for therapeutic purposes, monoclonal antibodies whose heavy chains terminate with a glycine are often α -amidated (Skulj, Pezdirec, Gaser, Kreft, & Zorec, 2014); elimination of the low levels of PAM expressed in the mammalian cells used to produce these monoclonal antibodies eliminates their α -amidation (Skulj et al., 2014). Dipeptides, X-Gly, are poor PHM substrates, exhibiting low V_{\max}/K_m ratios (Wilcox et al., 1999) and, in general, longer peptide substrates exhibit higher V_{\max}/K_m ratios (Chew et al., 2005). Modeling of *N*- α -acetyl-3,5-diodotyrosylglycine into the structure of PHMcc indicates that the carboxylate of the glycy residue forms a salt-bridge with Arg²⁴⁰ (Prigge, Mains, Eipper, & Amzel, 2000). This arginine is highly conserved across PHM sequences and likely provides a rationale for the importance of the free carboxylate for substrate binding. PHMcc is comprised of two domains of approximately the same size that are connected by a single strand; the single copper atom bound to each domain is poised at the edge facing the solvent-filled cleft separating the domains (Prigge et al., 1997). The interiors of both domains are hydrophobic and the copper atoms are separated by 11Å (Prigge et al., 1997). Upon reduction, there is virtually no change in PHMcc structure (Prigge et al., 2004). The large, hydrophobic interdomain region of PHMcc is necessary for the amidation of relatively large peptide substrates and is consistent with a preference for penultimate hydrophobic amino acids in the substrate. How electron transfer and radical stability are accomplished in the large, solvent accessible interdomain region of PHM are amongst the unanswered questions about PHM catalysis. A PHM structure with a large peptide substrate bound might provide insight about these issues.

Strikingly, PHM and PAL will accept a wide variety of non-peptide substrates possessing a moiety that is equivalent to the C-terminal glycine, including the *N*-acylglycines, hippurate (*N*-benzoylglycine) and substituted hippurates (Merkler et al., 2008), and the bile acid glycine conjugates (King et al., 2000). A carbinolamide intermediate is known to form as these substrates are converted into the amide and glyoxylate. Consistent with the preference for a hydrophobic penultimate amino acid for peptide substrates, the V_{\max}/K_m ratio for *N*-acylglycine amidation increases as acyl-chain length increases. Early studies by Kaptopodis and May demonstrated that PHM could also catalyze sulfoxidation, amine *N*-dealkylation, and *O*

-dealkylation (A.G. Katopodis & S.W. May, 1990) (**Fig. 2B, C, D**). The PHM catalyzed *O*-dealkylation of the imino-oxy acetates yields the corresponding oximes and glyoxylate in a PAL-independent manner (N.R. McIntyre, Lowe, Battistini, Leahy, & Merkler, 2016; N. R. McIntyre, Lowe, & Merkler, 2009; Schade, Kotthaus, Hungeling, Kotthaus, & Clement, 2009). Scorpions, which rely on a variety of amidated peptides to attack the ion channels of their prey, have retained separate genes encoding integral membrane PAM, soluble PHM and soluble PAL (Delgado-Prudencio, Possani, Becerril, & Ortiz, 2019), leading the authors to speculate that the PHM secreted in scorpion venom might catalyze some of these additional reactions. A complete understanding of PHM catalysis must account for the alternative reactions catalyzed by this enzyme.

A series of N-blocked Phe-(D,L)-homocysteine peptide analogs were synthesized and evaluated as PAM inhibitors (Erion, Tan, Wong, & Jeng, 1994; Jeng, Fujimoto, Chou, Tan, & Erion, 1997) (**Fig. 3A**). Members of this series with IC₅₀ values of 8-15 nM possess an *N*-hydrocinnamoyl, *N*-2-naphthoyl, or *N*-2-indolyl moiety conjugated to the Phe residue. Synthesis of the benzyl ester of *N*-2-naphthoyl-L-phenylalanyl-(D,L)-homocysteine as a prodrug increased the IC₅₀ from 10 to 8,000 nM, but 10 μM free carboxylate or benzyl ester showed approximately 50% inhibition of PHM in cultured rat dorsal root ganglion neurons, (Jeng et al., 1997). The potency of this series of compounds mirrors the substrate specificity information from the glycine-extended peptides. The partial PHM inhibition observed in the dorsal root ganglion neurons identifies cell penetration as a challenge for the future clinical use of these compounds. The disappointing results observed using dorsal root ganglion neurons for the benzyl ester suggest that the benzyl ester is not the optimal prodrug, at least, for this series of PHM inhibitors. The tight-binding of the homocysteine peptide analogs likely results from the coordination of sulfur atom to Cu_M (**Fig. 3B**). The binding of thiorphan (K_M = 82 μM), tiopronin (K_M = 33 μM), and captopril (K_i ~100 μM), all compounds with a free sulfhydryl group, was attributed to coordination of the sulfur atom to one of PHM-bound Cu(II) atoms (N. R. McIntyre, Lowe, Chew, Owen, & Merkler, 2006; Mueller, Driscoll, & Eipper, 1999).

One class of compounds investigated as PHM inhibitors are the glycolates, R-CO-O-CH₂-COOH; the R-groups described include an acyl group (short- and long-chain), a benzoyl group, a phenylacetyl-group, an *N*-acetyl-amino acid, and *N*-acetyl-peptides (Barratt et al., 2004; F. Cao et al., 2011; A.G. Katopodis & S.W. May, 1990; Morris et al., 2012; Ping et al., 1995) (**Fig. 3A**). The glycolates are analogs of the glycine-extended substrates and were clear substrate analogs for consideration as inhibitors. The glycolates are PHM-specific and have no effect on PAL (Moore & May, 1999). We have summarized the data for the glycolate and structurally similar inhibitors in **Table 1A** and, from these data, we note the following trends. A number of the glycolate inhibitors exhibit K_i or IC₅₀ values <5 μM, meaning that this class of compounds represent a good starting point for the future development of higher affinity PHM inhibitors. While the C-terminal glycolate analogs of two α-amidated peptide precursors (oxytocin and calcitonin) exhibit IC₅₀ values of 2-12 μM, the structurally simpler *O*-acylglycolates, like *O*-lauroylglycolate (CH₃-(CH₂)₁₀-CO-O-CH₂-COOH), exhibit sub-μM affinities, against PHM proteins from cultured mammalian cells. Thus, a future tight-binding glycolate inhibitor might be possible without the complications associated with peptide synthesis. Another encouraging observation is the differences in affinity between PHM proteins for some of the glycolate inhibitors. For example, *O*-lauroylglycolate exhibits an IC₅₀ of 60 nM for human lung cancer PHM and an IC₅₀ of 35 μM for frog PHM, a ratio of IC₅₀ values of ~600. This suggests that tissue-, organism-, or disease-state-specific PHM inhibitors may be possible. The stereochemistry of the penultimate group has a dramatic effect on the affinity of the glycolate inhibitors. The ratio of the K_i values for the D-amino acid glycolate/L-amino acid glycolate is 40-50 for the *N*-acetyl-D-Phe-O-CH₂-COOH/*N*-acetyl-L-Phe-O-CH₂-COOH pair and for the *N*-acetyl-D-Leu-O-CH₂-COOH/*N*-acetyl-L-Leu-O-CH₂-COOH pair. An even stronger preference for an L-amino acid at the penultimate position was identified for the glycine-extended peptide substrates; *N*-Acetyl-L-Phe-Gly binds to PHM [?]700-fold more tightly than *N*-acetyl-D-Phe-Gly (Ping et al., 1995). Future tight-binding glycolate inhibitors could take advantage of the stereochemical preference for the penultimate position.

Cao et al. (F. Cao et al., 2011) synthesized a series of thioglycolates, R-CO-S-CH₂-COOH, to determine any effect of the sulfur atom on the affinity of the PHM inhibitor (**Fig. 3A** and **Table 1B**). In general,

the thioglycolates inhibit with higher affinity, with a decrease in the IC_{50} value by a factor of 2-25 relative to the corresponding glycolate. The greatest difference was observed for the *N*-acetyl-Phe-O-CH₂-COOH/*N*-acetyl-Phe-S-CH₂-COOH pair, with a ratio of IC_{50} values > 80 for frog PHM. Again, these results suggest that sufficient differences exist between PHM proteins to enable the design of an inhibitor targeted against a specific PHM.

PHM inhibition by the glycolates and the thioglycolates coupled with the discovery of *N*-acylglycines as PHM substrates lead to a structurally related set of PHM inhibitors, the *S*-(thiocarbonyl)thioglycolates (R-CS-S-CH₂-COOH) (**Fig. 3A** and **Table 1C**). *S*-(Thiolauroyl)thioglycolate, CH₃-(CH₂)₁₀-CS-S-CH₂-COOH, is the tightest binding inhibitor within this set of compounds, with a $K_i = 540$ nM, a relatively high affinity for a ground-state, substrate analog. A direct comparison between structurally related glycolates, thioglycolates, and the *S*-(thiocarbonyl)thioglycolates to define whether PHM binds most tightly to either the -CS-S- group, the -CO-S- group, or the -CO-O- group is not possible because the PHM protein used each study was different. For example, the differences in K_i or IC_{50} values for C₆H₅-CH₂-CS-S-CH₂-COOH (8 μ M for rat MTC PHM), C₆H₅-CH₂-CO-S-CH₂-COOH, (20 μ M for frog PHM), and C₆H₅-CH₂-CO-O-CH₂-COOH (2 μ M for human H889 cell PHM) might result from the different sources of PHM. The *S*-(thiocarbonyl)thioglycolate study does provide a direct comparison of K_i values between rat MTC PHM and German cockroach PHM for six of these inhibitors; differences were found, with C₆H₅-CH₂-CS-S-CH₂-COOH binding to cockroach PHM with ~10-fold higher affinity (**Table 1**). Again, these data point towards the future development of inhibitors targeting PHM in a species-specific manner.

In their focus on the synthesis of PHM inactivators designed to capture the substrate-based radical that likely forms during catalysis, the Vederas group identified new PHM inhibitors. Included in this group are D-Phe-L-Phe- α -cyclopropylglycine (Andrews, O'Callaghan, & Vederas, 1997) and tripeptides with a C-terminal D- or L-styrylglycine (Zabriskie et al., 1992) (**Fig. 3A**). PHM is inactivated by *trans*-4-phenyl-3-buteonate (*trans*-styrylacetate) (A. F. Bradbury, Mistry, Roos, & Smyth, 1990). The cyclopropylglycine-containing tripeptide does not inactivate PHM and binds with low affinity, $K_i > 5$ mM, additional evidence that PHM does not readily accommodate any substitution on the glycyl α -carbon. None of the styrylglycine-containing peptides inactivate PHM, but all inhibit with IC_{50} values of 100 to 450 μ M. This indicates that the styryl moiety must be optimally positioned in the PHM active site for inactivation to occur. Even a small repositioning of the styryl group eliminates its ability to inactivate PHM; neither *N*-acetyl-D- nor *N*-acetyl-L-styrylglycine inactivates PHM. Little difference in the IC_{50} values of the L- and D-styrylglycine containing inhibitors was found (Zabriskie et al., 1992). Thus, the importance of the stereochemistry at the penultimate position for PHM affinity found for the glycine-extended substrates and the glycolate inhibitors (Ping et al., 1995) was not observed for the styrylglycine-containing inhibitors. The significance of these differences is currently unknown and could be resolved by *in silico* modeling or crystallographic analysis of appropriate PHM-ligand complexes. The information gained from such studies could prove useful for the future design of tight-binding PHM inhibitors.

One clear outcome from the work carried out on PHM inhibitors is preference for hydrophobicity in groups attached to the glycine or glycine analog. Consistent with this observation are reports of hydrophobic organic acids that inhibit PAM with relatively low affinity. Examples from this group of PHM inhibitors include 1-(carboxymethyl)-3,5-diphenyl-2-methylbenzene ($IC_{50} = 550$ μ M) (Cutler et al., 1998), urocanic acid ($K_I = 10$ mM) (Merkler et al., 2008), and 4-pentenoic acid ($IC_{50} = 60$ mM) (Rhodes & Honsinger, 1993).

One other PHM inhibitor, mimosine (**Fig.3A**), differs from the rest. Mimosine is a toxic, heterocyclic, non-protein amino acid produced by plants (Nguyen & Tawata, 2016), which is known to inhibit the mechanistically-related enzyme, dopamine β -monooxygenase (Hashiguchi & Takahashi, 1977). Mimosine is competitive vs. ascorbate, determined by varying the concentrations of mimosine and ascorbate at one-fixed concentration of glycine-extended substrate, yielding a K_I of 4.0 μ M for mimosine (Miller et al., 1992). *In silico* modeling reveals that mimosine binds to PHM between the two copper atoms, forming hydrogen bonds to the backbone carbonyls of Pro-268 and Leu-270 and to the side chain amide of Gln-269 (**Fig.3A**). Additional *in silico* modeling suggests that mimosine and ascorbate bind at approximately the same site in

PHM (**Fig.3B**), consistent with mimosine being competitive vs. ascorbate. The appropriate attachment of mimosine (or a mimosine analog) to an inhibitor that binds to the peptide site in PHM could yield a bifunctional inhibitor of high potency (**Fig.3C**).

3B. PAL inhibitors: The metals bound to PAL remain bound during its purification. Like PHM, metal chelators can inhibit PAL by removing the bound Zn(II). Activity is restored to inactive apo-PAL by the addition of Zn(II) and other divalent metal ions (Bell et al., 1997). Unlike PHM, PAL does not exhibit stereochemical preference at the penultimate position; the kinetic constants for *N*-acetyl-L-Phe- α -hydroxyglycine and *N*-acetyl-D-Phe- α -hydroxyglycine are very similar (Ping et al., 1995). C-Terminal pyruvate-extended amino acids, R-CO-CH₂-CO-COOH, are inhibitors of PAL with K_i values >15 μ M. *N*-Acetyl-L-Phe-pyruvate is the best PAL inhibitor reported, with a K_i of 0.24 μ M. The pyruvate-extended amino acids weakly inhibit PHM, being competitive with ascorbate and a \sim 100-fold lower potency (Mounier et al., 1997). The related compound, C₆H₅-CH=CH-SO-CH₂-COOH (*trans* isomer), shows no inhibition or inactivation of PHM at 3 mM, but was not evaluated as a PAL inhibitor (Casara, Banzhorn, Philippo, Chanal, & Danzin, 1996). We are not aware of any other work to develop a PAL-specific inhibitor.

4. PHM Inactivators

There are important differences between an enzyme inhibitor (I) and an enzyme inactivator (IN). An inhibitor forms a reversible enzyme-inhibitor complex: E + I \rightleftharpoons E-I. An inhibitor shows no time-dependence in the percent inhibition at any inhibitor concentration. An inactivator forms an irreversible enzyme-inactivator complex (E-IN)* from the reversible E-IN complex: E + IN \rightleftharpoons E-IN \rightarrow (E-IN)*. Typically, the (E-IN)* complex results from the formation of a covalent bond between the inactivator and the enzyme. At any concentration of inactivator, the degree of inhibition increases over time as higher concentrations of (E-IN)* accumulate (Morrison & Stone, 1985).

The first report of a PHM inactivator was *trans*-4-phenyl-3-butenolate (PBA) (A. F. Bradbury, Mistry, Roos, et al., 1990). In addition to PBA and ring-substituted PBAs (Langella et al., 2010), other PHM inactivators include *trans*-styrylthioacetate (Casara et al., 1996), 2-[(phenylethynyl)thio]acetate (Casara et al., 1996), acrylates (Foster, Oldham, & May, 2011; A. G. Katopodis & S. W. May, 1990; Rhodes & Honsinger, 1993), monoethyl fumarate (A. G. Katopodis & S. W. May, 1990), 2-, 3-, and 2,4-alkenoates (Rhodes & Honsinger, 1993), cinnamate and ring-substituted cinnamates (A. F. Bradbury, Mistry, & Smyth, 1990; N.R. McIntyre et al., 2016), *N*-formyl amides (Klinge, Cheng, Zabriske, & Vederas, 1994), and peptides with a C-terminal vinylglycine (Zabriske et al., 1994). Treatment of cultured mammalian cells and rats with PBA inhibits PHM activity and the biosynthesis of α -amidated peptides (Abou-Mohamed et al., 2000; Ogonowski et al., 1997). The methyl ester prodrug of PBA was \sim 10-fold more effective than PBA in inhibiting the growth of tumorigenic rat liver epithelial cells (WB-*Ras* cells) (Sunman, Foster, Folse, May, & Matesic, 2004).

Most of the PHM inactivators were designed as mechanism-based inactivators to trap the radical intermediate that is likely to form during catalysis (F. Cao & Easton, 2013; Cowley et al., 2016). These are mechanism-based inactivators because O₂ and ascorbate are required for inactivation and peptide substrates protect against inactivation. However, the chemistry of inactivation is unclear (F. Cao & Easton, 2013; Driscoll et al., 2000; N.R. McIntyre et al., 2016; Zabriske et al., 1992). As expected PAL is unaffected by the PHM inactivators (N.R. McIntyre et al., 2016; Moore & May, 1999). Attempts to label PHM using either a ¹⁴C-labeled or fluorescently-tagged inactivator have been unsuccessful, with the exception of one early study of PBA-mediated inactivation, in which the labeled protein was not subjected to peptide mapping. Peptide-mapping of cinnamate-inactivated PHM showed no differences compared to the untreated control (N.R. McIntyre et al., 2016). We are unaware of any PAL-specific inactivators.

5. What have we learned towards the design of a PAM targeted therapeutic

When viewed collectively, patterns emerge from the studies of the PHM inhibitors and inactivators. Clearly, PHM prefers a free carboxylate conjugated to a hydrophobic moiety positioned as close as possible to the penultimate amino acid for the glycine-extended peptide substrates. Incorporation of a sulfur-containing group that can coordinate with one of the PHM-bound copper atoms would likely increase binding affin-

ity. Other possibilities to increase binding affinity in future PHM inhibitors/inactivators would be to link mimosine (or a mimosine analog) to a compound that binds at the peptide site to create a bifunctional inhibitor. The identification of a putative mimosine-binding site (**Fig.3C**) in conjunction with the published PHM structures (W. A. Francisco, Blackburn, & Klinman, 2003; Prigge et al., 2004) and the wealth of structure-activity data for PHM substrates and inhibitors provide an excellent starting point for *in silico* modeling of high affinity PHM inhibitors or inactivators. Another possible bifunctional inhibitor could include a copper-chelator linked to a compound that binds at the peptide site in PHM. Inhibitors/inactivators with high affinity unique to PAM could possess a PHM binder linked to the PAL-specific pyruvate-extended amino acids. Without a structure for bifunctional PAM, a series of compounds with different length spacers between the PHM inhibitor and the PAL inhibitor would be required to define the compound with the highest affinity for PAM. The appropriate incorporation of mimosine into a PAM inhibitor could yield a tight-binding trifunctional inhibitor with a group that binds into the mimosine site of PHM, the peptide site of PHM, and the substrate site of PAL.

The development of a high affinity PHM (or PAM) inhibitor will encounter significant hurdles that could hinder clinical use. One concern is delivery. PHM is found within the lumen of the secretory pathway (Kumar, Mains, et al., 2016), a challenging site for drug delivery. As discussed above, the clinical use of a PHM inhibitor is likely dependent upon a molecular zip code for the secretory system or the surface of specific cell types. Perhaps an engineered version of the Shiga toxin might enable the delivery of a high affinity PHM/PAM inhibitor to the secretory system (Luginbuehl, Meier, Kovar, & Rohrer, 2018). Another concern is the diversity of the amidated products produced *in vivo* by PHM. The inhibition of PHM would produce unselective blocking of the biosynthesis of many amidated peptides and lipids. One solution to this concern is not a PHM inhibitor, but the development of inhibitor that binds selectively and with high affinity to one glycine-extended substrate, which inhibits the amidation of only one PHM substrate (Weiss, McIntyre, McLaughlin, & Merkler, 2006).

The inactivators require at least one *trans*-olefinic bond positioned β - to a carboxylate for the most efficient inactivation. Inactivators with the highest affinity have the inactivating moiety attached to the C-terminus of a peptide or a hydrophobic group like a phenyl group. The hydrophobic group must be appropriately spaced away from the inactivating species for the highest affinity, exactly as was observed for the glycine-extended substrates and the PHM inhibitors. Again, the available structure-activity data and PHM structures provide an excellent backdrop for the *in silico* design of high affinity inactivators. The decoration of such high affinity inactivators with the appropriate imaging reagent could yield a PHM-specific imaging reagent, demonstrating that the molecular targeting was in fact successful. Another application would be decoration with biotin to enable the profiling of PHM similar to the activity-based profiling strategies developed by Cravatt et al. (Cravatt, Wright, & Kozarich, 2008). However, additional research is required to unravel uncertainties in the PHM inactivation chemistry to fully exploit the development of a PHM-specific imaging reagent.

6. The bigger picture – PAM from green algae to human

PAM and other granule membrane proteins are deposited on the cell surface during the process of exocytosis (**Fig.4A**). Immature granules contain PAM and soluble cargo proteins. During the maturation process, cargo proteins are further modified; the granules move from the Golgi region towards the cell surface and acquire the cytosolic proteins needed to make them responsive to the right stimuli. Any PAM protein in the granule membrane appears on the cell surface as the soluble cargo proteins are released into extracellular space (**Fig.4A**, Exocytosis). Under normal conditions, PAM is rapidly removed from the plasma membrane (**Fig.4A**, Endocytosis) and returned to the secretory granules via a complex series of steps through the endocytic pathway. Clathrin coated vesicles mediate the removal of PAM from the cell surface and detailed studies of PAM trafficking in corticotrope tumor cells have tracked the internalized protein as it moves from early endosomes into late endosomes (Bäck et al., 2017; Rajagopal, Stone, Francone, Mains, & Eipper, 2009). Whether the PAM protein that exits late endosomes is degraded in lysosomes or is recycled to immature granules (**Fig.4A**) is determined by O-linked sugars attached to the linker region between PHM and PAL, by a conserved pH-sensitive region that follows PHMcc and by the phosphorylation status of the PAM cytosolic

domain (Rajagopal et al., 2009; Rao, Zavala, Deb Roy, Mains, & Eipper, 2019; K. Vishwanatha, Bäck, Mains, & Eipper, 2014; K. S. Vishwanatha, Bäck, Lam, Mains, & Eipper, 2016). Targeting its O-linked sugars or its pH-sensitive region could provide a means of preventing the return of PAM to the secretory pathway.

Compounds that stimulate the secretion of peptide hormones and neurotransmitters stored in granules increase the amount of PAM protein delivered to the plasma membrane (**Fig.4A** , lightning bolt). Tumor cells often secrete autocrine growth factors, many of which are amidated peptides (Jimenez et al., 2001). The appearance of PAM on the surface of these cells provides an opportunity for cell-impermeant PHM inhibitors or PAM-targeted tags that can be tracked *in vivo* to bind to its ectodomain and enter the endocytic pathway along with PAM. Internalization of a PHM inhibitor would be expected to reduce the synthesis of bioactive amidated growth factors while ectodomain antibodies or active-site targeted tags might facilitate tumor cell localization (**Fig.4A**). An inactive prodrug might be expected to bind to PAM while it is exposed on the cell surface, with drug activation designed to occur in the lower pH environments encountered during endocytic trafficking. As discussed above, with an understanding of the structure of bifunctional PAM, the design of dual function drugs (targeting PHM and PAL) might provide a tool capable of preventing the endocytic trafficking needed to return PAM to the secretory pathway. Outside of its active sites, the sequences of insect and human PHM may be divergent enough to design species specific inhibitors useable as insecticides.

Phylogenetic studies have suggested several different situations in which controlling PAM activity or localization could be beneficial. While the active sites of *C. reinhardtii* PAM (CrPAM) include each of the residues identified as essential in vertebrate PHM and PAL, this unicellular green alga does not store peptides in secretory granules (**Fig.4B**). CrPAM is localized to the membranes of the Golgi complex, as observed in vertebrates, but the presence of two prominent motile cilia made it clear that CrPAM is also localized to the ciliary membrane (**Fig.4B**) (Kumar, Blaby-Haas, et al., 2016). Unlike the enzymes used by *C. reinhardtii* to respond to the need for various nutrients, neither PAM activity nor PAM protein is secreted through the classical secretory pathway (Kumar et al., 2017; Luxmi et al., 2019). In vertebrate systems, endoproteolytic cleavages that separate the PHM and PAL domains from the transmembrane domain result in their secretion under both basal and stimulated conditions (Kumar, Mains, et al., 2016). During vegetative growth, *C. reinhardtii* rely on bioactive ectosomes released from their cilia (**Fig.4B** , Inset) to destroy the mother cell wall (Wood, Huang, Diener, & Rosenbaum, 2013). Ciliary ectosomes also play a key role during sexual reproduction in *C. reinhardtii* (M. Cao et al., 2015). Active CrPAM is present in mating ectosomes, along with its amidated products, one of which acts as a chemomodulator (Luxmi et al., 2019) (**Fig.4B**). PAM was subsequently identified in the ciliary membranes of vertebrate motile and sensory cilia (Kumar et al., 2017). The formation of ciliary ectosomes (**Fig.4B**) is topologically similar to the formation of intraluminal vesicles in the endocytic pathway (**Fig.4C**). Fusion of a multivesicular body containing ILVs results in their release as exosomes (**Fig.4C**). PAM has been identified in intraluminal vesicles (Bäck et al., 2017) and in exosomes isolated from human saliva and urine (Gonzalez-Begne et al., 2009; Principe et al., 2013; Z. Wang, Hill, Luther, Hachey, & Schey, 2012). Much remains to be learned about the role played by ectosomes/exosomes, but the presence of PAM in these structures offers a means of isolating and characterizing that subset.

When PAM expression was reduced in *C. reinhardtii* , the cells were unable to form cilia (**Fig.4D**) (Kumar et al., 2017). Instead of their normal pair of motile 12 μm long cilia (**Fig.4B**), PAM knockdown cells had ciliary stubs containing microtubule fragments. The ciliary axoneme did not form and the appearance of the transition zone was altered. In humans, fully 5% of the genome is thought to be involved in the formation and function of cilia and ciliopathies are associated with many congenital diseases including obesity, blindness and kidney disease (Engle, Bansal, Antonellis, & Berbari, 2021; Leroy et al., 2021; Nager et al., 2017). The effects of reducing PAM levels on ciliogenesis are species specific, but occur in planaria, zebrafish and mouse (**Fig.4D**). The hypothalamic neurons that produce proopiomelanocortin must have cilia and the melanocortin 4 receptor must be localized to those cilia in order to regulate appetite normally (Y. Wang et al., 2021). Much remains to be learned about the sensory and signaling roles of cilia and tools that allow the manipulation of ciliary PAM would be of great utility in these studies.

It is now known that the PAM protein has several non-catalytic functions (**Fig.4E**). Regulated intramem-

brane proteolysis of PAM releases a soluble fragment of its cytosolic domain (sfCD). Nuclear accumulation of sfCD depends on its phosphorylation status and is thought to contribute to the ability of PAM to alter gene expression in a tissue-specific manner (Rajagopal et al., 2009). In atrial myocytes, which express PAM at levels higher than those observed in other cells, the PAM protein (whether active or inactive) plays an essential role in the formation of atrial granules, which store natriuretic peptides (Bäck, Luxmi, Powers, Mains, & Eipper, 2020). Instead of its catalytic activity, the ability of PAM to function as a re-usable luminal cargo receptor for proatrial natriuretic peptides early in the secretory pathway is thought to be essential to its ability to support granulogenesis.

Acknowledgments

We thank the many lab members and colleagues over several decades whose insights and hard work contributed to these studies. Supported by: NIH R01-DK032948 and R01-DK032949 (BAE, REM); NIH R01-GM051293 (BAE, Stephen M. King); The Daniel Schwartzberg Fund (BAE, REM); NIH R21-GM140390 and R15-GM073659 (DJM); Eppley Foundation for Research (DJM); Milheim Foundation for Cancer Research (DJM); Unigene Laboratories, Inc. (DJM). *Figure Legends*

Figure 1. Peptide processing basics, the amidation reaction and amidation enzymes. **A.** The preprotein peptide precursor is processed by endoproteases (prohormone convertases such as PCSK1, PCSK2, PCSK3) and an exoprotease (such as CPE or CPD) to present to PAM the immediate precursor (peptide-Gly), which yields the final amidated peptide product. **B.** The two-step PAM reaction. Step 1 is performed by the first enzyme, PHM, and Step 2 is performed by PAL. The two copper residues bound to PHM are reduced by ascorbate, peptide and dioxygen bind to the reduced enzyme, a proton is abstracted and the peptide is hydroxylated by PHM. The N-C bond is then cleaved by PAL, liberating the α -amidated peptide plus glyoxalate. **C.** Proteins used for enzymology and structural determination. In mammals, two major forms of PAM are the bifunctional membrane enzymes PAM1 and PAM2, each with a transmembrane domain (TMD) and short cytoplasmic domain (CD). The proteins used thus far for structural analyses are the recombinant catalytic cores, PHMcc and PALcc. The reactions have been studied with purified natural proteins from various sources, recombinant pure rat PHMcc and PALcc, and with two pure recombinant bifunctional proteins, rat PHM-PAL820s and Type A a-AE. References given in the text. Residues (using NP_037132.2): PHMcc (42-356), PALcc (498-820, lacking N-glycosylation [S767A]), PHM-PAL820s (42-820 lacking Exon 16 [residues 393-498], lacking N-glycosylation [S767A]), Type A PAM (27-820 lacking Exon 16).

Figure 2. The Reactions Catalyzed by PHM and PAL. Bifunctional PAM is comprised of the two separate catalytic units, PHM and PAL (Reaction A). Steps 4 and 5 in reaction A represent a collection of steps in the PHM mechanism (Cowley et al., 2016; Prigge et al., 2000; Wu et al., 2019). Reactions B and C catalyzed by PHM, any involvement of PAL in reactions B and C have not been specifically addressed. The S-dealkylation reaction, shown in reaction B, is consistent with the finding of glyoxylate as a minor product during the sulfoxidation reaction. A sulfoxide/glyoxylate ratio of 8 was reported, but no mercaptan was found (A.G. Katopodis & S.W. May, 1990). The formation of glyoxylate from the imino-oxy acetate (reaction D, bottom reaction) is PAL-independent.

Figure 3. Ligands that Bind to PHM and PAL. **A.** Structures for the inhibitors and activators discussed in this review. **B.** *In silico* model for the interaction of PHMcc with N-hydrocinnamoyl-L-Phe-L-homocysteine, $IC_{50} = 10$ nM (compound #22 from (Erion et al., 1994)). **C.** *In silico* model for the interaction of PHMcc and mimosine. **D.** *In silico* model for the interaction better PHMcc and mimosine or ascorbate. **E.** *In silico* model of the interaction of PHMcc with N- α -acetyl-3,5-diodotyrosylglycine and ascorbate. Our model is based on the published model of the PHMcc complexed with the same dipeptide (Prigge et al., 2000). The copper atoms are shown in brown (B-E) and the PHMcc backbone is either gray (B) or light green (C-E). For Panels C-E, the amino acid ligands for the copper atoms are in blue and binding site amino acid side chains are in dark green. The colors for the ligands are as follows: N-hydrocinnamoyl-L-Phe-L-Phe is purple (B), mimosine is purple (C and D), ascorbate is brownish-yellow (D and E), and N- α -acetyl-3,5-diodotyrosylglycine is gray (E). The *in silico* models were generated using AutoDock Vina. *N*

-Hydrocinnamoyl-L-Phe-L-homocysteine was docked using the flexible side chain method, with the ligand covalently attached and then modeled as a flexible residue (Bianco, Forli, Goodsell, & Olson, 2016).

Figure 4. A. PAM trafficking in neuroendocrine cells. As newly synthesized PAM and soluble cargo proteins exit the *trans*-Golgi, they accumulate in immature secretory granules. Granule maturation involves vesicular trafficking and acquisition of the cytosolic proteins needed to respond to secretagogues. Upon fusion of the secretory granule membrane with the plasma membrane, soluble content proteins are released and membrane PAM appears on the cell surface. Clathrin-mediated endocytosis means that less than 5% of the PAM protein in a cell typically resides on the plasma membrane. PAM retrieved from the cell surface can be degraded or returned to the secretory pathway for re-use.**B.** In *C. reinhardtii*, PAM is localized to the Golgi Complex, small vesicular structures and the ciliary membrane. The ciliary budding process that generates ectosomes is illustrated, with the localization of CrPAM and one of its amidated products (Cre03.g20450) illustrated (Luxmi et al., 2018; Luxmi et al., 2019).**C.** The movement of PAM through late endosomes and into the intraluminal vesicles (ILVs) that form in multivesicular bodies (MVBs) was determined using ectodomain antibodies (Bäck et al., 2017; Rajagopal, Mains, & Eipper, 2012). Upon fusion with the plasma membrane, PAM-containing exosomes are released. **D.** The species-specific roles of PAM in ciliogenesis are summarized.**E.** The non-catalytic effects of PAM are summarized. The ability of PAM to alter gene expression is thought to require the generation of sfCD through γ -secretase-catalyzed regulated intramembrane proteolysis (RIP) (Rajagopal, Stone, Mains, & Eipper, 2010). Neither the ability of PAM to support the formation of atrial granules or its ability to interact with actin require its catalytic activity.

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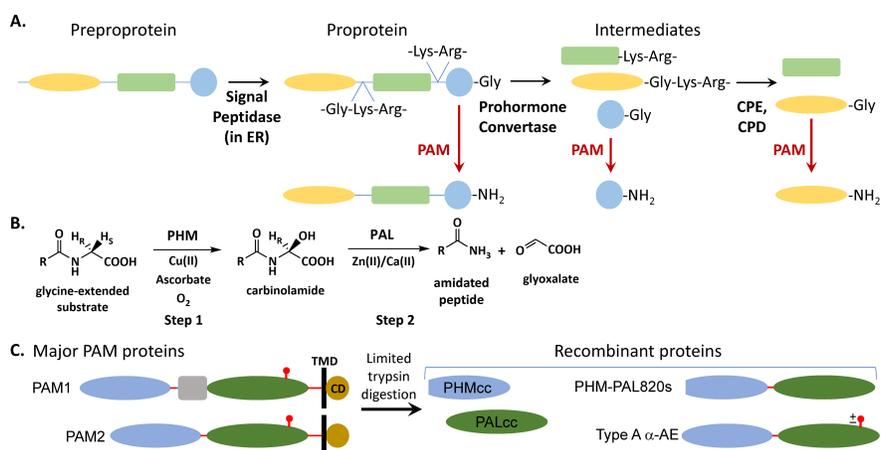
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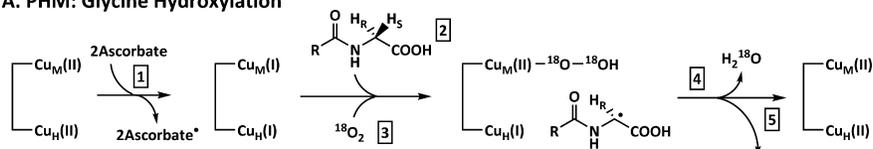
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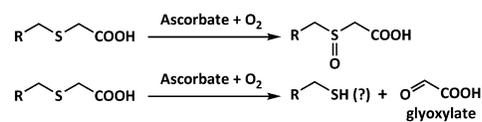
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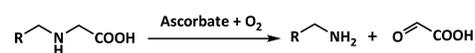
A. PHM: Glycine Hydroxylation



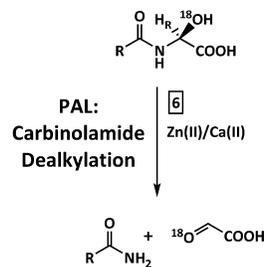
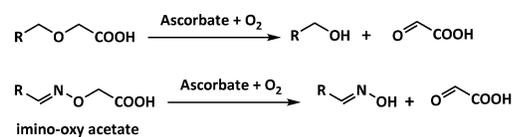
B. PHM: Sulfoxidation (and S-Dealkylation)



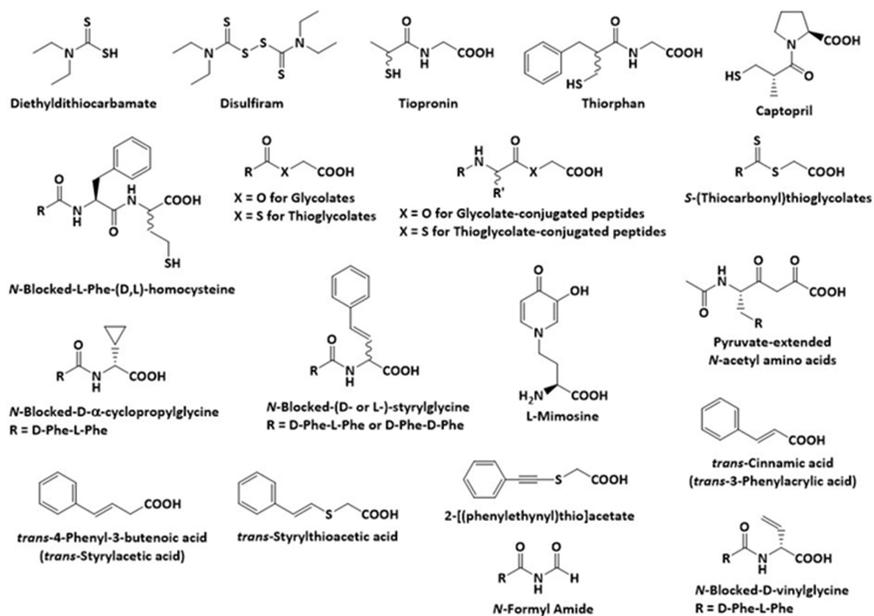
C. PHM: Amine N-Dealkylation



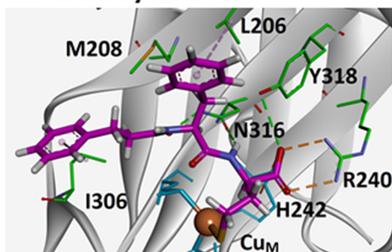
D. PHM: O-Dealkylation



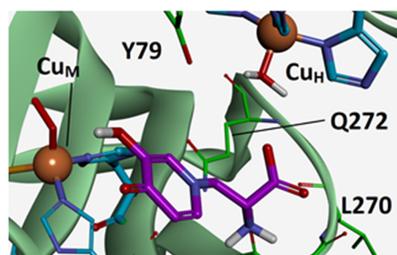
A. PHM Inhibitor and Inactivator Structures



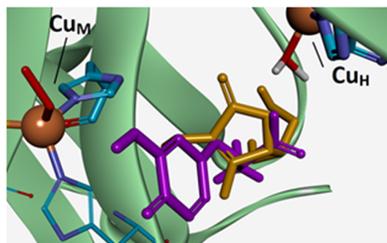
B. Hydrocinnamoyl-L-Phe-L-Homocysteine



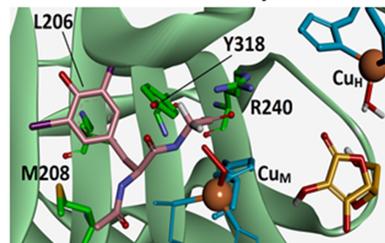
C. Mimosine

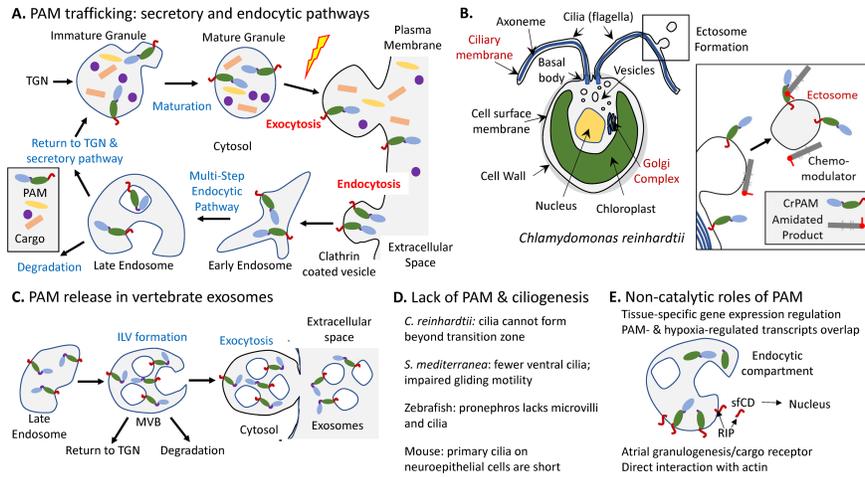


D. Mimosine and Ascorbate



E. Ascorbate and Peptide





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