Peptidylglycine α-Hydroxylating Monooxygenase: Active Site Residues, Disulfide Linkages, and a Two-Domain Model of the Catalytic Core†

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ABSTRACT: Peptidylglycine α-hydroxylating monooxygenase (PHM) is a copper, ascorbate, and molecular oxygen dependent enzyme that catalyzes the first step leading to the C-terminal amidation of glycine-extended peptides. The catalytic core of PHM (PHMcc), refined to residues 42−356 of the PHM protein, was expressed at high levels in CHO (DG44) (*dhfr*$^-$) cells. PHMcc has 10 cysteine residues involved in 5 disulfide linkages. Endoprotease Lys-C digestion of purified PHMcc under nonreducing conditions cleaved the protein at Lys^{219}, indicating that the protein consists of separable N- and C-terminal domains with internal disulfide linkages, that are connected by an exposed linker region. Disulfide-linked peptides generated by sequential CNBr and pepsin treatment of radiolabeled PHMcc were separated by reverse phase HPLC and identified by Edman degradation. Three disulfide linkages occur in the N-terminal domain (Cys^{47}−Cys^{186}, Cys^{81}−Cys^{126}, and Cys^{114}−Cys^{131}), along with three of the His residues critical to catalytic activity (His^{107}, His^{108}, and His^{172}). Two disulfide linkages (Cys^{227}−Cys^{334} and Cys^{293}−Cys^{315}) occur in the C-terminal domain, along with the remaining two essential His residues (His^{242}, His^{244}) and Met^{314}, thought to be essential in binding one of the two nonequivalent copper atoms. Substitution of Tyr^{79} or Tyr^{318} with Phe increased the K_m of PHM for its peptidylglycine substrate without affecting the V_{max}. Replacement of Glu^{313} with Asp increased the K_m 8-fold and decreased the k_{cat} 7-fold, again identifying this region of the C-terminal domain as critical to catalytic activity. Taking into account information on the copper ligands in PHM, we propose a two-domain model with a copper site in each domain that allows spatial proximity between previously described copper ligands and residues identified as catalytically important.

Carboxy-terminal amidation is an essential posttranslational modification for the bioactivity of numerous neuronal and endocrine peptides. Amidation of glycine-extended precursors is catalyzed by a bifunctional enzyme, peptidylglycine α-amidating monooxygenase (PAM),[1] in a two-step reaction (Figure 1) (Bradbury & Smyth, 1991; Eipper et al., 1992b; Katopodis & May, 1990a; Takahashi et al., 1990; Young & Tamburini, 1989). The first step, hydroxylation at the α-carbon of the terminal glycine, is catalyzed by peptidylglycine α-hydroxylating monooxygenase (PHM) (EC 1.14.17.3), a copper, ascorbate, and molecular oxygen dependent enzyme, and yields a peptidyl-α-hydroxyglycine intermediate. The second step, dealkylation of this intermediate, is catalyzed by peptidyl-α-hydroxyglycine-α-amidating lyase (PAL) (EC 4.3.2.5), a divalent metal ion dependent enzyme, leaving the carboxamide moiety at the C-terminus of the bioactive peptide and generating glyoxylate.

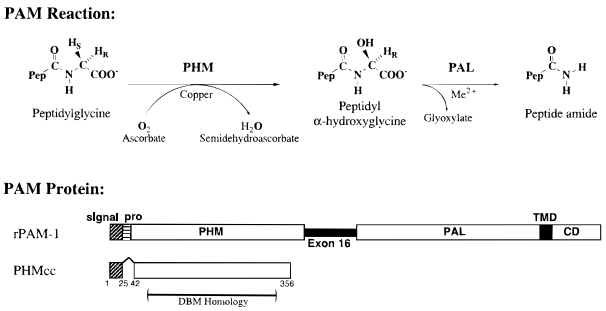

FIGURE 1: PAM-catalyzed reaction and PAM proteins. The reactions catalyzed by PHM and PAL are shown. The structures of PAM-1 and PHMcc are compared; the region of homology between PHM and DBM is indicated. TMD, transmembrane domain; CD, COOH-terminal domain.

PAM is the only enzyme known to catalyze the C-terminal amidation of peptides (Eipper et al., 1992b), and deletion of the PHM gene in *Drosophila* is lethal (Kolhekar et al., 1997). In the rat, several isoforms of this enzyme exist due to tissue-specific and developmentally regulated alternate splicing of the single-copy PAM gene (Iwasaki et al., 1993; Eipper et al., 1992a). The largest rat PAM is a membrane-associated bifunctional protein of 120 kDa which can undergo endoproteolytic cleavage to yield a soluble PHM domain (45 kDa) and a membrane-associated (70 kDa) or soluble (50 kDa) PAL domain (Eipper et al., 1992a; Milgram et al., 1992). The individual PHM and PAL domains are each catalytically competent (Milgram et al., 1992; Eipper et al., 1991, 1995; Katopodis & May, 1990b).

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[1] Abbreviations: DBM, dopamine β-monooxygenase; CNBr, cyanogen bromide; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; rPAM, rat PAM.

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The PHM reaction exhibits ping-pong kinetics (Gilligan et al., 1989); two ascorbate molecules first reduce the two enzyme-bound copper atoms with the formation of two molecules of semidehydroascorbate (Merkler et al., 1992). The enzyme-bound copper atoms are nonequivalent (Boswell et al., 1996; Merkler et al., 1995a) and undergo redox cycling (Freeman et al., 1993). PHM catalyzes the stereospecific abstraction of the glycyl *pro-S* hydrogen (Ramer et al., 1988) with the proposed formation of an α-carbon-centered glycyl radical which is subsequently hydroxylated (Erion et al., 1994; Zabriskie et al., 1992). The catalytic mechanism of PHM has been probed using mutagenesis (Eipper et al., 1995; Yonekura et al., 1996) and inhibitors (Bradbury et al., 1990; Casara et al., 1996; Erion et al., 1994; Katopodis & May, 1990b; Kizer et al., 1986; Klinge et al., 1994; Merkler et al., 1995a,b; Mounier et al., 1997; Rhodes & Honsinger, 1993; Zabriskie et al., 1992), but the key enzyme functional groups and the catalytic mechanism are not yet clear.

PHM and dopamine β-monooxygenase (DBM) (EC 1.14.17.1) catalyze similar reactions (Eipper et al., 1983) and exhibit 32% sequence identity over a 291 amino acid region (Southan & Kruse, 1989). Recent spectroscopic analyses of oxidized PHM show that the two copper atoms are liganded to five histidinyl nitrogens and three oxygens (from solvent, Asp, or Glu) (Boswell et al., 1996). Upon reduction of Cu^{2+} to Cu^{+}, one of the oxygen ligands is replaced by a sulfur ligand; mutagenesis studies suggest that the sulfur ligand is from the side chain of Met^{314} (Boswell et al., 1996; Eipper et al., 1995). Site-directed mutagenesis has demonstrated the importance of all five His residues conserved between rat PHM and rat DBM as potential copper ligands (Eipper et al., 1995; Yonekura et al., 1996). Mechanism-based inhibition studies of DBM identified two catalytically important Tyr residues (DeWolf et al., 1988; Farrington et al., 1990). Based on this information, we previously mutated one of these residues, Tyr^{79}, and observed a slight increase in the K_m of PHM for the tripeptide substrate (Eipper et al., 1995).

A monofunctional rat PHM protein engineered to terminate at residue 382 (40 kDa) was purified from hEK-293 cells, and limited proteolytic cleavage studies had shown that a 35 kDa PHM protein that started with residue 42 was fully active (Husten et al., 1993). The PHM/DBM homology region includes rPAM(53−343). In order to facilitate further study of PHM, we created a smaller PHM protein that was stable and active; the 35 kDa catalytic core of PHM (PHMcc) includes amino acids 42−356 of rat PAM-1. Using endoproteolytic cleavage, we defined non-disulfide-linked N- and C-terminal domains of PHMcc. We established the five disulfide linkages in PHM by sequence analysis of peptides derived from PHM metabolically labeled with [^{35}S]Cys and [^{3}H]Trp. We analyzed PHMcc proteins bearing mutations in potential active site residues and copper ligands. Based on the disulfide linkages, domain boundaries, and active site mutants, we propose a model for the active site of PHM.

EXPERIMENTAL PROCEDURES

Construction and Purification of the Catalytic Core of PHM (PHMcc). The PHMcc gene was constructed by PCR mutagenesis of pBS.ΔProPHM382s (Eipper et al., 1995) and expressed using the pCIS.2CXXNH vector (Tausk et al., 1992). Unless specifically indicated, all numbering of amino acid residues is based on rat PAM-1 (Stoffers et al., 1989). The DNA sequences encoding rPAM-1(36−41) and rPAM-1 (357−382) were deleted separately, and transient expression of the truncated proteins was carried out in hEK-293 cells (Jordan et al., 1996) to ensure the integrity of the enzyme. The 5′ and 3′ truncations of the PHM gene were combined to create pBS.PHMcc. The pCIS.PHMcc vector was constructed and stably transfected into CHO (DG44) (*dhfr*$^{-}$) cells (created by Dr. L. A. Chasin, Columbia University, and kindly provided by Dr. Iris Lindberg, Louisiana State University) as described (Wernicke & Will, 1992) using αMEM (GIBCO) (growth medium lacking nucleotides) supplemented with 10% dialyzed calf serum (GIBCO) and selected on the basis of secreted PHM activity.

Synthesis and Purification of PHMcc. The stably transfected CHO.PHMcc cells (clone 6α) were grown in 850 cm^2 roller bottles (Falcon) in αMEM supplemented with 10% dialyzed calf serum under CO_2. Upon reaching confluence, the cells were rinsed and placed into 250 mL of serum-free αMEM containing 1.25 μg/mL insulin and 0.1 μg/mL transferrin. The spent medium was collected daily for 5 days, and the cells were then placed into growth medium for 2 days. This cycle was repeated for 5−6 weeks. Upon removal from the cells, medium was sterile-filtered and phenylmethylsulfonyl fluoride (0.3 mg/mL) was added. Proteins in 5 L of spent medium were precipitated by addition of 0.44 g/mL $(NH_4)_2SO_4$. The precipitate was dissolved in 4 mL of 0.5 M $(NH_4)_2SO_4$, 20 mM NaTES (pH 7.4) and applied to a Sephadex G75-120 (Sigma) column (2 × 90 cm) equilibrated with the same buffer. Fractions containing PHMcc were pooled, and PHMcc was further purified on a Mono Q column (HR5/5) on the FPLC as described (Eipper et al., 1995). Further purification was achieved by HPLC on a C_4 column (Vydac, 4.6 × 250 mm) using a gradient of CH_3CN in 0.1% TFA. The protein was judged to be >95% pure using SDS−PAGE to fractionate the purified protein and Coomassie Brilliant Blue R-250 to visualize the protein. Using complete amino acid analysis, the extinction coefficient at 280 nm of PHMcc, $\epsilon^{280}_{1mg/mL}$, was determined to be 1.29 in 0.1 N HCl. PHMcc (100 μg/100 μL) was assayed for free thiol groups in 3.2 M guanidine hydrochloride using Ellman's reagent (Sigma) at 412 nm as described (Riddles et al., 1983).

Synthesis of [^{35}S]Cys- and [^{3}H]Trp-Labeled PHMcc. A 90% confluent 2 cm^2 well of CHO-6α cells was preincubated for 30 min with serum-free DMEM (GIBCO) lacking Met and Cys. The Cys residues were metabolically labeled by incubation of the cells with DMEM containing 250 μCi/mL [^{35}S]-L-cysteine (ICN Biochemicals; 1030 mCi/μmol), 120 μM Met, 1.25 μg/mL insulin, and 0.1 μg/mL transferrin for 4 h. [^{3}H]Trp-labeled PHMcc was synthesized by metabolic labeling of a 90% confluent 75 cm^2 flask of CHO-6α cells for 21 h in DMEM containing 50 μCi/mL [5-^{3}H]-L-tryptophan (NEN Research Products; 25 mCi/μmol), 120 μM Met, 1.25 μg/mL insulin, and 0.1 μg/mL transferrin. [^{35}S]-Cys- and [^{3}H]Trp-labeled PHMcc were purified by ammonium sulfate precipitation followed by hydrophobic interaction chromatography as described (Eipper et al., 1995). Further purification was carried out on the C_4 column as described above following addition of 0.5 mg of purified PHMcc.

Endoprotease Lys-C Digestion. Limited digestion of purified PHMcc was carried out with Endo Lys-C (Boehringer Mannheim) (1:100 w/w) in 100 mM Tris-HCl (pH 8.0). Aliquots were removed at various time intervals and placed at −80 °C. The aliquots were subjected to SDS−PAGE under nonreducing or reducing conditions (5% β-mercaptoethanol) and transferred onto Immobilon P membranes as described (Husten & Eipper, 1991). Endo Lys-C-generated PHMcc fragments were visualized using Coomassie dye or Western blot analysis. Western blot analysis was performed using rabbit polyclonal antibodies to peptides contained within PHMcc and ECL (Amersham) (Eipper et al., 1995); JH100 recognizes rPAM (298−315) (Ouafik et al., 1989), and JH246 recognizes rPAM(116−131) (Husten & Eipper, 1991).

Cyanogen Bromide and Pepsin Cleavage. Purified [^{35}S]-Cys-labeled PHMcc (10 mg; 6×10^6 cpm) was cleaved with CNBr in 70% TFA as described (Lee & Shively, 1990). The CNBr-generated peptides could not be separated by reverse phase or ion exchange HPLC, so further endoproteolytic digestion was carried out with pepsin since pepsin digestion could be performed under acidic conditions to prevent disulfide interchange. The digest was brought close to dryness under vacuum and dispersed in 0.1% TFA for cleavage with pepsin. Pepsin (Sigma) (total 1:100 w/w) was added in three aliquots, and digestion was carried out at 37 °C for 48 h. The digest was stored in aliquots at −80 °C until further analysis.

Reverse Phase HPLC. The entire CNBr/pepsin digest of [^{35}S]Cys-labeled PHMcc was chromatographed in 5 runs on a Waters C_{18} μBondapak column (3.9 × 300 mm) equilibrated with 0.1% TFA, 3% CH_3CN on Beckman System Gold. Buffers A and B were composed of 0.1% TFA in water and 0.1% TFA in CH_3CN, respectively. A linear gradient of 0.33% B/min between 3 and 37%B was used to separate the peptides. A flow rate of 0.5 mL/min was maintained, and absorbance measurements were carried out at 220 nm. Fractions (0.5 mL) with disulfide bond-containing peptides were identified by liquid scintillation spectroscopy of aliquots using a Beckman LS2800 liquid scintillation counter. Fractions containing radiolabeled peptides were pooled and further chromatographed on the same column using heptafluorobutyric acid (HFBA) (Fluka Chemicals) as an ion pairing agent. Radiolabeled peptides were prepared for sequence analysis by chromatography on the same column equilibrated with 0.1% TFA and eluted using a shallow gradient of CH_3CN.

Endoprotease Glu-C Digestion. Peptides containing [^{3}H]Trp and [^{35}S]Cys were purified by RP-HPLC in the CH_3CN/TFA system, pooled, and brought to dryness in a vacuum centrifuge. The peptides were dissolved in 100 mM ammonium acetate (pH 4.0), 2.5% CH_3CN and digested with Endo Glu-C (Boehringer Mannheim) (1 μg/25 μL) for 18 h at 37 °C. The digests were immediately chromatographed on the C_{18} column using the CH_3CN/TFA system.

Edman Sequencing. Isolated radiolabeled fractions from HPLC were evaporated to dryness, dissolved in 50 μL of 0.1% TFA, 50% CH_3CN, and applied to the Applied Biosystems Model 477A pulsed-liquid instrument for sequence analysis with on-line HPLC identification of phenylthiohydantoins. Disulfide-linked peptides were revealed as paired sequences which were identified by comparison with the known sequence of the rat PAM protein.

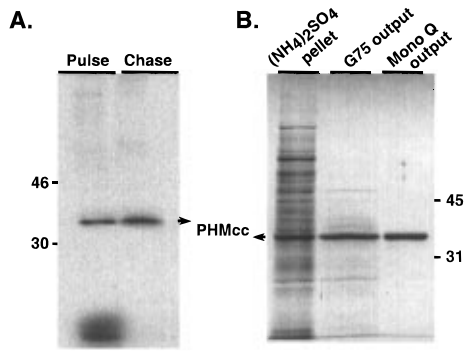

FIGURE 2: PHMcc produced by CHO-6α cells. (A) The Cys residues of PHMcc were metabolically labeled by incubation of CHO-6α cells with DMEM/F12 containing [^{35}S]-L-Cys for 4 h (Pulse). This medium was replaced with nonradioactive complete serum-free medium for 4 h (Chase). Aliquots representing equal fractions of the Pulse and Chase media were subjected to SDS−PAGE and fluorography. (B) Proteins transferred to Immobilon were visualized with Coomassie Brilliant Blue R-250. SDS−PAGE of input to G75 column [$(NH_4)_2SO_4$ pellet], input to Mono Q column (G75 output), and purified PHMcc (Mono Q output).

Generation and Analysis of Mutant PHMcc Proteins. Individual mutations were introduced into the pBS.PHMcc plasmid using the PCR-based method of gene splicing by overlap extension as described (Horton et al., 1990), creating PHMcc with His107Ala, His107Ala/His108Ala, His172Ala, His242Ala, Glu313Asp, Met314Cys, Met314His, or Tyr318Phe (Eipper et al., 1995). Stable CHO cell lines producing each of the mutant PHMcc proteins were generated as described above. Mutant proteins were characterized using biosynthetic labeling, substrate affinity chromatography, PHM enzyme assays, and Western blot analysis as described (Eipper et al., 1995). To provide an accurate estimate of the amount of PHMcc in a sample of medium, aliquots of medium containing a range of PHM activity were subjected to Western blot analysis; following visualization of PHM by ECL, films were scanned, and the signals were quantified. Aliquots of medium yielding signals in the linear range of the film were used to calculate the amount of PHM protein present.

RESULTS

PHMcc. The smallest PHM protein expressed previously was rPAM(36−359) with the wild-type signal sequence (Eipper et al., 1995). The effects of further N-terminal (start with Ser^{42}) and C-terminal (end with Val^{356}) truncations were evaluated separately. Transient transfection of hEK-293 cells with each construct resulted in the secretion of active enzyme, indicating that neither truncation eliminated the catalytic ability of PHM. Hence, the signal sequence [rPAM(1−25)], which is required for secretion, was appended to the catalytic region of PHM [rPAM(42−356)], and this putative PHMcc was expressed in a Chinese hamster ovary cell line lacking the dihydrofolate reductase gene, CHO (DG44). An overproducing cell line, CHO-6α, secreting large amounts of PHM activity was selected. The CHO-6α cells were labeled with [^{35}S]Cys for 4 h and chased with complete medium for 4 more h, and the medium was subjected to SDS−PAGE and fluorography without any purification (Figure 2A). Precipitation of total cellular proteins with trichloroacetic acid indicates that PHMcc represents 1.5% of the total [^{35}Cys]-labeled protein synthesized by CHO-6α cells. More than 90% of the total [^{35}Cys]-labeled secreted protein is PHMcc.

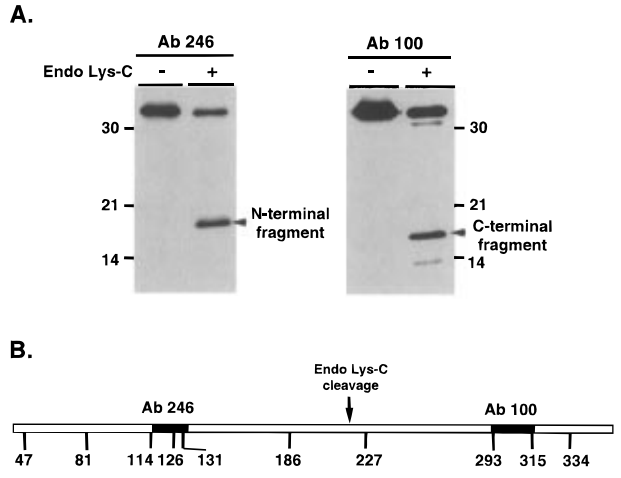

FIGURE 3: Identification of N- and C-terminal domains of PHMcc. (A) The locations of the 10 Cys residues contained within PHMcc and the peptides used to generate the antibodies used in this experiment are indicated. The location of Lys^{219}, identified by Edman degradation as the site of Endo Lys-C cleavage, is shown. (B) PHMcc subjected to limited endoproteolysis with Endo Lys-C (Experimental Procedures) was fractionated by SDS−PAGE under nonreducing conditions, transferred to Immobilon, and visualized with antisera generated to two different PHMcc peptides. The experiment was replicated 3 times with similar results.

Purification of PHMcc. For large-scale production, the CHO-6α cells were grown in roller bottles in complete serum-free medium where approximately 1 mg/L of PHMcc protein was secreted per day. PHMcc was concentrated and then purified from the spent medium in two steps. As the culture became older, the medium had more contaminating proteins from ruptured cells; however, greater than 95% purity was achieved after size-exclusion and Mono Q chromatography (Figure 2B). Analysis of the kinetics of hydroxylation of [^{125}I]-Ac-Tyr-Val-Gly by purified PHMcc yielded k_{cat} and K_m values (100 s^{-1} and 13 μM, respectively) comparable to values reported previously for the slightly larger PHM proteins (Eipper et al., 1995). The optimum pH for hydroxylation by PHMcc was 5.5, which is the same pH optimum observed for PHM when assayed as part of PAM-3 (Husten et al., 1993).

Domains of PHMcc. We used limited endoproteolysis to begin to investigate the topology of PHMcc. Antisera generated to peptides contained within the N-terminal and C-terminal regions of PHMcc (Figure 3A) were used to identify digestion products. The endoprotease reactions were carried out in nondenaturing and oxidizing conditions for varying times with several different enzymes. The reaction products were subjected to SDS−PAGE under nonreducing conditions followed by Western blot analysis using both antipeptide antibodies. Digestion of native PHMcc with endoprotease Lys-C gave the most useful set of products (Figure 3B). PHMcc was cleaved into two major fragments with molecular masses of 18.5 and 16.5 kDa. Based on antibody cross-reactivity, the 18.5 kDa fragment was identified as the N-terminal domain, and the 16.5 kDa fragment was identified as the C-terminal domain. The N-terminal sequence of the 16.5 kDa C-terminal domain was determined by Edman degradation; a single sequence was read for 10 residues [V^{220}VNADIS(C)QYK], indicating that endoproteolytic cleavage had occurred after Lys^{219}. Thus, Lys^{219} resides in an exposed surface region linking the two domains. Measurement of free thiol groups in PHMcc using Ellman's reagent shows that all 10 Cys residues are involved in disulfide linkages. Disulfide bonds do not link the two domains; thus, the six Cys residues in the N-terminal domain and the four Cys residues in the C-terminal domain form disulfide linkages within each domain.

Identification of Disulfide Linkages. In order to facilitate the identification of peptides containing cysteine residues, we prepared [^{35}S]Cys-labeled PHMcc (6×10^6 cpm) (Figure 2A) and mixed the purified radiolabeled PHMcc with 10 mg of purified PHMcc. PHMcc is quite resistant to proteolytic digestion, and, after testing numerous endoproteases in an attempt to achieve near-complete fragmentation under acidic pH conditions, we chose to use CNBr for our initial fragmentation step. Maintenance of acidic pH during fragmentation was essential since disulfide linkages can exchange at neutral or basic pH (Smith & Zhou, 1990). PHMcc has 16 Met residues, predicting a complex pattern of CNBr peptides even after reduction. The CNBr cleavage did not go to completion, as evidenced by SDS−PAGE of reduced samples (data not shown), and the CNBr-generated disulfide-linked fragments tended to aggregate, precluding their separation by reverse phase, size-exclusion, or anion exchange chromatography.

Although pepsin is a fairly nonspecific endoprotease, its acidic pH optimum led us to use it to further fragment the CNBr peptides. When fractionated by RP-HPLC, the CNBr/pepsin digest of [^{35}S]Cys-labeled PHMcc yielded a complex mixture of peptides (Figure 4A). Fractions were collected, and aliquots were counted to determine the location of the [^{35}S]Cys-containing peptides. The profile of radiolabeled peptides was rather complex, reflecting partial cleavage with CNBr and nonspecific cleavages with pepsin; 10 peptide pools were made, and the [^{35}S]Cys-labeled peptides were subjected to further purification. Following fractionation in the presence of heptafluorobutyric acid, an ion pairing agent (Bennett, 1991), the radiolabeled peptides were further purified using a shallow gradient of CH_3CN in the presence of TFA to remove the heptafluorobutyric acid.

Fractions containing purified [^{35}S]Cys-labeled peptides were subjected to Edman degradation, and the sequences obtained are shown in Figure 4B. Purified disulfide-linked peptides would be expected to yield equivalent amounts of two sequences, and a representative example of amino acid yields is presented in Table 1. Based on the sequences obtained, the Cys^{227}−Cys^{334} and Cys^{293}−Cys^{315} linkages were assigned, demonstrating the presence of two disulfide linkages within the C-terminal domain of PHMcc. In the N-terminal domain, a peptide containing the Cys^{47}−Cys^{186} linkage was identified (Figure 4B). Peptides containing the other four Cys residues located within the N-terminal domain (Cys^{81}, Cys^{114}, Cys^{126}, and Cys^{131}) were identified in pools G and J; three N-terminal sequences were detected indicating that no cleavage had occurred between Cys^{126} and Cys^{131} (Figure 4B). Therefore, the four cysteine residues are linked by two disulfide bonds, but the only linkage that could be eliminated based on these data is Cys^{126} to Cys^{131}. We attempted to separate the two cysteine residues in the C^{126}-DEGTC^{131} sequence using endoprotease Glu-C at pH 7.5, but sequencing showed a mixture of products due to scrambling of the disulfides.

We therefore reduced the pH for endoprotease Glu-C cleavage to 4.0. Given the complexity of the CNBr/pepsin digest of PHMcc and the likelihood that endoprotease Glu-C

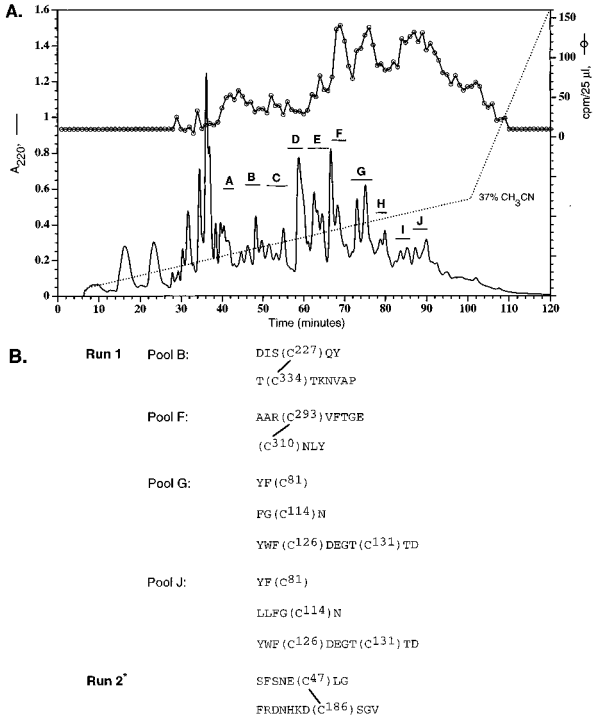

FIGURE 4: Analysis of CNBr and pepsin-digested [^{35}S]Cys-labeled PHMcc. (A) PHMcc (10 mg; 6×10^6 cpm) treated sequentially with CNBr and pepsin was fractionated on a C_{18} column using a gradient of acetonitrile; the absorbance was monitored at 220 nm, and an aliquot was subjected to liquid scintillation spectroscopy. The fractions containing [^{35}S]Cys-labeled peptides were pooled as shown (A−J). Peptides in pools A−J were further fractionated as described under Experimental Procedures until absorbance and radiolabel were coincident in a sharp peak. The peptide yields were typically 50−100 pmol starting with 300 pmol of PHMcc. (B) Disulfide-linked peptide sequences obtained by Edman degradation. (C) Cys residues are not observed. *Disulfide-bridged peptides eluted at ~23% CH_3CN. HPLC data for run 2 have not been shown.

Table 1: Sequence Analysis of Pool F[a]

cycle #	sequence 1	yield	sequence 2	yield
1	Cys^{315}	−	Ala^{290}	171
2	Asn	136	Ala	195
3	Leu	83	Arg	131
4	Tyr	13	Cys^{293}	−
5			Val	127
6			Phe	125
7			Thr	75
8			Gly	137

[a] The peptides in pool F were purified and subjected to Edman degradation. Except when Cys residues occur, two major amino acids were identified in each of the first four steps; yields are reported. Given the sequence of PHMcc, these amino acids could only derive from the unique sequence pair of disulfide-linked peptides indicated. Sequence 1 terminated after the fourth cycle, indicating that cleavage had occurred at this position.

would cleave less efficiently at pH 4.0, we sought a way to facilitate identification of even small amounts of the desired cleavage products. Tryptophan, one of the essential amino acids, can be used to prepare radiolabeled PHMcc, and Trp^{124}, one of only three Trp residues in PHMcc, is separated from Cys^{126} by a single residue and is not separated from Cys^{126} by the CNBr/pepsin protocol employed (Figure 4B). The only [^{3}H]Trp/[^{35}S]Cys-labeled material in a digest of PHMcc should be derived from Cys^{126}, and the ratio of ^{3}H to ^{35}S should indicate whether the essential cleavage has occurred. We biosynthetically labeled PHMcc with [^{3}H]Trp, mixed the

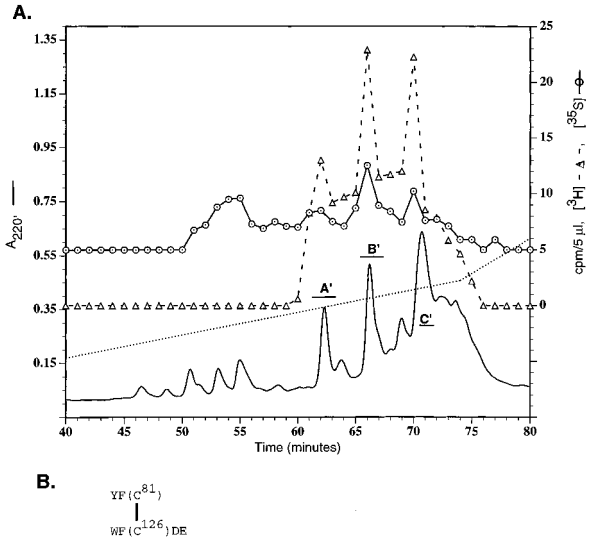

FIGURE 5: Analysis of [^{35}S]Cys/[^{3}H]Trp-labeled PHMcc. (A) Purified PHMcc radiolabeled with [^{35}S]Cys and [^{3}H]Trp was cleaved with CNBr and pepsin and fractionated as described in Figure 4A; peptides eluting in the region equivalent to pool J were further digested with Endo Glu-C, and the digest was again applied to the C_{18} column and eluted with an acetonitrile gradient as indicated. Absorbance was monitored at 220 nm, and aliquots were subject to liquid scintillation spectroscopy with energy windows adjusted to distinguish ^{3}H from ^{35}S. (B) N-Terminal sequences obtained by Edman degradation of purified peptides from pool B′.

[^{3}H]Trp-labeled PHMcc (1.4×10^6 cpm) with [^{35}S]Cys-labeled PHMcc (0.8×10^6 cpm), and purified PHMcc (4 mg) and digested the mixture with CNBr followed by pepsin. The digest was chromatographed on the C_{18} column (as shown in Figure 4A), and fractions corresponding to pool J were pooled for treatment with endoprotease Glu-C at pH 4.0 in ammonium acetate buffer. The product peptides were again chromatographed on the C_{18} column (Figure 5A). As indicated, only peptides in pools A′, B′, and C′ contained both radiolabels; peptides in these three pools were purified to homogeneity using heptafluorobutyric acid and then TFA. The sequences of the [^{3}H]Trp- and [^{35}S]Cys-labeled peptides are shown in Figure 5B. Positive identification of the Cys^{81}−Cys^{126}-linked peptides allows assignment of the single remaining disulfide to Cys^{114}−Cys^{131} since no free thiols were detected in PHMcc and sequence analysis of a single component in pools G and J yielded three sequences containing Cys^{81}, Cys^{114}, Cys^{126}, and Cys^{131}.

Role of Conserved Histidine Residues in Establishing the Structure of PHMcc. There are only five His residues conserved among PHM and DBM; based on mutagenesis studies of PHM, each of these five conserved His residues is essential for catalytic activity (Eipper et al., 1995; Yonekura et al., 1996). Mutation of His^{108} or His^{244} to Ala yielded proteins that were synthesized and secreted with normal kinetics (Eipper et al., 1995). In contrast, mutation of His^{242} to Ala reportedly yielded an unstable protein (Yonekura et al., 1996). In order to evaluate the role of the His residues in maintaining the structure of PHMcc, we mutated the remaining conserved His residues in PHMcc (His^{107}, His^{172}, His^{242}) to Ala and evaluated both the ability of stably transfected CHO cells to secrete the newly synthesized protein and the ability of each mutant protein to bind to a peptidylglycine substrate affinity resin (Figure 6).

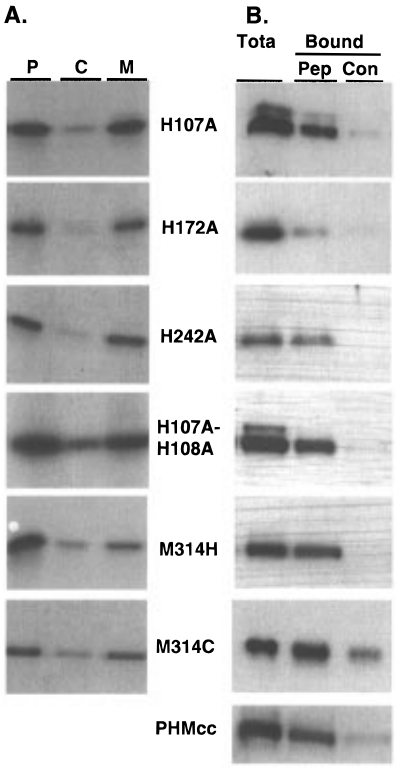

FIGURE 6: Mutation of conserved His and Met[314] residues has no effect on *in vivo* protein folding and substrate binding. (A) Duplicate wells of CHO cells stably expressing PHMcc with His[107], His[172], and His[242] or both His[107] and His[108] replaced by Ala, or Met[314] replaced by Cys or His, were incubated with medium containing [^{35}S]Met for 15 min; cells in one well were harvested immediately (P), and cells in the other well were incubated an additional 6 h in medium lacking labeled Met before harvesting cells (C) and medium (M). Aliquots representing an equal fraction of all three samples were immunoprecipitated with antiserum to PHM and analyzed by SDS−PAGE and fluorography. (B) Spent medium from control cells (PHMcc) or from each of the PHMcc mutants was incubated with Affi-Gel resin to which D-Tyr-Trp-Gly was covalently bound (Pep) or to the same resin lacking peptide (Con). Equal fractions of the input and the material bound to the resin were fractionated by SDS−PAGE, and PHM was visualized using the antipeptide antibody JH246 directed to a region unaffected by the mutations. Both experiments were replicated at least twice for each cell line.

As expected, mutation of any of the conserved His residues to Ala completely eliminated the catalytic activity of PHMcc (data not shown). However, no alteration was observed in the rate at which stably transfected CHO cells secreted the newly synthesized mutant proteins or the efficiency with which newly synthesized mutant PHMcc was recovered in the medium (Figure 6A). Even PHMcc bearing two mutations (His[107]−His[108]) was secreted efficiently with normal kinetics. This result indicates that the His residues, although essential for catalytic activity, are not essential for the folding of newly synthesized PHMcc into a form that allows exit of the protein from the endoplasmic reticulum.

To evaluate better the structural integrity of the mutant proteins, their ability to bind to a peptidylglycine substrate affinity resin was compared to that of PHMcc (Figure 6B). Each mutant protein was able to bind to the substrate affinity resin. If the His residues are essential for the binding of Cu^{2+} to PHM, they are not essential for the binding of peptidylglycine substrate.

Active Site Mutants of PHMcc. Studies on the mechanism of the reaction catalyzed by DBM have indicated an important role for Tyr residues (DeWolf et al., 1988;

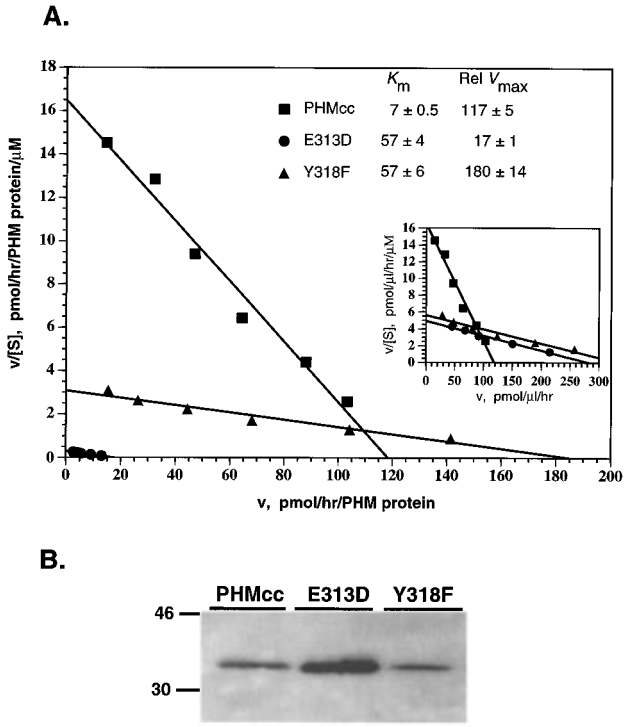

FIGURE 7: Kinetic analyses of the E313D and Y318F PHMcc mutant enzymes. (A) Eadie−Hofstee plot using the spent medium from stably transfected CHO cells expressing PHMcc or the two mutant proteins. Assays were carried out in duplicate at pH 5.0 in the presence of 0.5 μM $CuSO_4$ and 0.5 mM ascorbate; the concentration of Ac-Tyr-Val-Gly substrate was varied over at least a 3−5-fold range above and below the K_m. The data shown are the average of two independent determinations for each PHMcc protein and have been corrected for the amount of PHM protein in each sample. The inset shows the data prior to normalizing for amounts of protein. (B) Aliquots of the medium used for assays were subjected to Western blot analysis using an antipeptide antibody to an epitope distant from the site of any of the mutations. The samples shown yielded equal amounts of PHM activity when assayed at saturating substrate concentration. The actual assessment of protein concentration used to estimate V_{max} was made by analyzing additional dilutions of each sample.

Farrington et al., 1990); equivalent biochemical studies have not yet been conducted with PHM. We previously mutated one of the equivalent tyrosyl residues in PHM (Tyr[79]) to Phe and observed an increase in the K_m of the mutant PHM protein for peptidylglycine substrate (Eipper et al., 1995). Based on studies of DBM, Tyr[318] of PHM might also play a role in the active site, so we expressed PHMcc with Phe[318] in CHO cells. Our earlier studies demonstrated a critical role for Met[314] in PHM and suggested that it might be the source of the S ligand thought to interact with one of the copper atoms bound to PHM (Eipper et al., 1995). The amino acid sequence around Met[314] (Glu[313]-Met[314]-Cys[315]) is absolutely conserved in all known PHM and DBM proteins. To investigate a potential role for Glu[313], we generated CHO cells expressing PHMcc with Asp[313]. In addition, we expressed PHMcc containing His[314] or Cys[314], replacing Met[314] with other amino acids that might also interact with copper.

Our metabolic labeling paradigm showed that the E313D and Y318F mutant proteins were efficiently folded and secreted by the stably transfected cell lines (data not shown). Enzyme assays of spent media from the stably transfected CHO cells indicated that neither of these mutations completely eliminated the catalytic activity, allowing a more

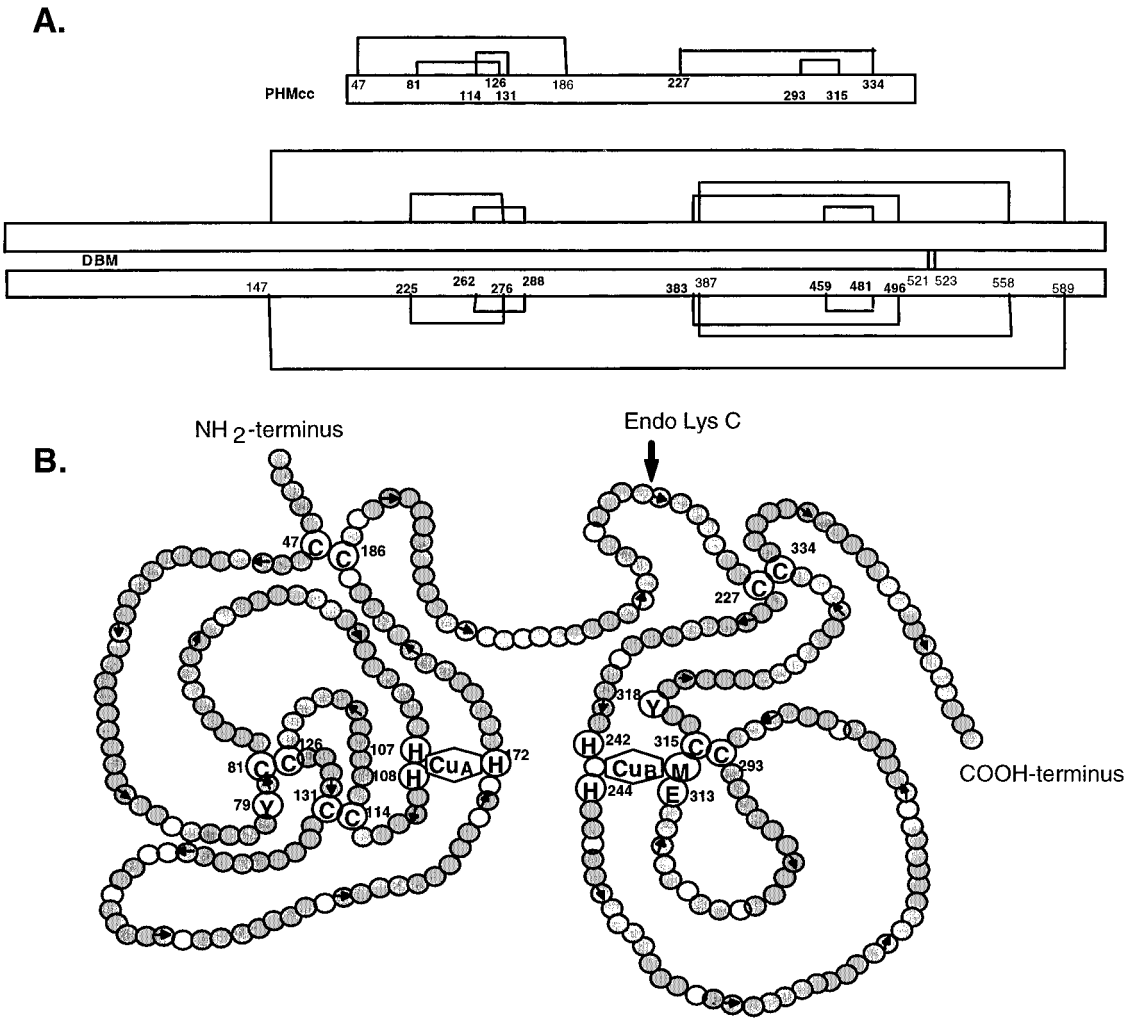

FIGURE 8: Proposed model of the active site of PHMcc. (A) The disulfide linkages established in this study for PHMcc are compared to those established for DBM (Robertson et al., 1994). (B) The constraints imposed upon PHMcc by the disulfide linkages determined in this study are shown; Cys residues are indicated (C). The site cleaved by digestion with Endo Lys-C is indicated. Since elimination of putative copper ligands does not dramatically alter the conformation of the protein, each copper is assigned to a separate domain of PHM. The ligands proposed for Cu_A and Cu_B are shown. Residues shown by mutagenesis to be important for catalysis are indicated. Arrows mark every tenth amino acid. All additional residues (a total of 42) absolutely conserved between PHM, DBM, and tyramine β-hydroxylase are shown by open circles; 25% of these conserved residues are Pro and Gly. Accession numbers for the PHM sequences used: *Xenopus laevis*, URXLA1, URXLA2; human, URHUAP; rat, URRTAP; bovine, P10731; equine, D29625; mouse, MMU79523; *Drosophila melanogaster*, AF006663. Accession numbers for the DBM sequences used: rat, Q05754; mouse, JC1346; bovine, A33650; human, S03020.

detailed analysis of their kinetic properties (Figure 7). The K_m values of the Ac-Tyr-Val-Gly substrate for the mutant Y318F and E313D enzymes were increased approximately 8-fold over that for wild type-PHMcc. In order to estimate the effect of each mutation on V_{max}, we determined the relative amount of PHMcc protein in each sample by Western blot analysis (Figure 7). The Y318F mutation had very little effect on the V_{max} for hydroxylation of the Ac-Tyr-Val-Gly substrate. The E313D mutation produced a 7-fold reduction in V_{max} compared to the wild-type PHMcc. Thus, the E313D substitution decreases k_{cat}/K_m by 50-fold.

Met^{314} in the active site of PHMcc is proposed to be a ligand to the reduced copper (Boswell et al., 1996), and we had previously shown that PHM bearing the M314I mutation was inactive (Eipper et al., 1995). We wished to investigate whether Met^{314} could be replaced by a His or a Cys residue. Substitution of Met^{314} with His or Cys did not restore catalytic activity, so we investigated the ability of these inactive mutants to fold and bind substrate (Figure 6). Both M314H and M314C PHMcc mutant proteins are secreted from CHO cells with close to normal kinetics, as seen by biosynthetic labeling, and both mutant proteins bind to the peptidylglycine resin efficiently.

DISCUSSION

By truncating the N- and C-termini of rat PHM, we have better defined the catalytic core of PHM, PHMcc. Our truncation studies indicate that the noncatalytic region at the N-terminus of PHM [rPAM(26−41)] is not essential even during the biosynthesis of PHM. Our earlier C-terminal truncation studies demonstrated that PHM that included all of the Cys residues but extended only to Thr^{335} was unstable, not secreted, and inactive while PHM that extended to Asp^{359} was fully active (Eipper et al., 1995). Here, we show that PHM truncated at Val^{356} is fully active. Interestingly, the naturally occurring monofunctional *Drosophila* PHM extends only six amino acid residues past Val^{356} (Kolhekar et al., 1997), suggesting that nature has confirmed our experimental conclusion. PHMcc was expressed at high levels in a CHO cell line, CHO-6α, allowing ready purification of milligram quantities of the enzyme. The availability of large amounts

of monofunctional PHM has facilitated further characterization of the structure of this enzyme.

PHMcc includes 10 cysteine residues (Figure 3A). Except for Cys^{47} and Cys^{186}, all of the cysteine residues are conserved between frog, human, rat, mouse, bovine, horse, and *Drosophila* PHM. The same eight cysteine residues are conserved in rat, mouse, bovine, and human DBM and in *Drosophila* tyramine β-hydroxylase (Monastirioti et al., 1996). All 10 of the cysteine residues in PHMcc are present in disulfide linkages, and the linkages connect $Cys^{47}-Cys^{186}$, $Cys^{81}-Cys^{126}$, $Cys^{114}-Cys^{131}$, $Cys^{227}-Cys^{334}$, and $Cys^{293}-Cys^{315}$ (Figure 8). Disulfide linkages occur at homologous positions in bovine DBM (Robertson et al., 1994); the Cys residues corresponding to linkage $Cys^{47}-Cys^{186}$ are absent from all species of DBM, *Drosophila* PHM, and *Drosophila* tyramine β-hydroxylase. DBM contains four additional disulfide linkages; two of the linkages are clearly interchain linkages and are outside the region exhibiting homology to PHMcc.

Limited endoproteolysis with Endo Lys-C under nonreducing conditions indicated that PHM has a two-domain structure (Figure 8) with the domains linked by a surface-accessible linker region containing Lys^{219}. Lys^{219} is located at the end of exon 9, which encodes no residues thought to be located near the active site. The assignment of disulfide linkages is consistent with a two-domain structure with three disulfide linkages within the N-terminal domain and two disulfide linkages within the C-terminal domain. Within each domain, a disulfide linkage connects a Cys residue near the beginning of the domain to a Cys residue near the end of the domain. The two additional disulfide linkages in the N-terminal domain should further constrain the region around Tyr^{79}.

The fact that each of the PHMcc proteins with a His mutation is efficiently synthesized and secreted and is still capable of binding peptidylglycine substrate suggests that each domain of PHM contains an independent copper binding site. If the copper binding sites involved ligands from both domains, one might expect mutation of a single His residue to affect protein folding and substrate binding ability. Three essential His residues and potential copper ligands (His^{107}, His^{108}, His^{172}) are located in the N-terminal domain. The remaining two essential His residues and potential copper ligands (His^{242} and His^{244}) along with the essential Met postulated to be a copper ligand (Met^{314}) are located in the C-terminal domain. Spectroscopic studies of bifunctional PAM, monofunctional PHM, and purified DBM indicate that there is a great deal of similarity in copper ligands (Blumberg et al., 1989; Boswell et al., 1996; Eipper et al., 1995; Reedy et al., 1995). The two copper atoms bound to each monomeric protein are not equivalent, with Cu_A bound by three His residues and Cu_B, the copper thought to interact with molecular oxygen, bound by two His residues and a Met upon reduction (Boswell et al., 1996). Taking this information into account, we propose that copper with the properties of Cu_A would be bound to the N-terminal domain while copper with the properties of Cu_B would be bound to the C-terminal domain of PHMcc (Figure 8).

Our model for PHMcc allows close proximity of the two copper sites in the enzyme active site. Our mutagenesis studies suggest that Glu^{313} and Tyr^{318} are localized near the active site. The role of Glu^{313} in the PHM reaction could be in peptidylglycine substrate binding, catalysis, and/or maintenance of the active site structural integrity, while Tyr^{318} is involved in binding of the substrate. Our earlier studies indicated that Tyr^{79}, situated in the N-terminal domain, is also involved in peptidylglycine substrate binding (Eipper et al., 1995). One of the mechanisms proposed for DBM involves formation of an active site tyrosyl radical (Tian et al., 1994). Our mutagenesis studies and enzyme kinetic studies suggest that neither Tyr^{79} nor Tyr^{318} plays an essential role in the PHM reaction. Although not identified as targets of mechanism-based inhibitors in studies of DBM (Farrington et al., 1990; DeWolf et al., 1988), there are two other completely conserved Tyr residues (Tyr^{321} and Tyr^{322}) in PHM and DBM; a role for these Tyr residues has not yet been investigated using mutagenesis. If there is an enzyme radical involved in the PHM reaction, conserved Gly, Cys, or Trp residues may need to be considered as sites for formation of that radical. All of these residues have been reported to form radicals in various other enzymes (Pedersen & Finazzi-Agro, 1993).

Comparison of all available PHM, DBM, and tyramine β-hydroxylase sequences indicates that 42 residues in the region equivalent to the 315 amino acid catalytic core are completely conserved. Eight of the conserved amino acids are Cys residues that form disulfide bridges, and six are His or Met residues involved in binding copper. Other conserved amino acids include residues likely to play a structural role (seven Gly residues and three Pro residues) along with aliphatic residues (seven Val, Leu, or Ala residues) likely to be buried in the interior of each domain. Of the four conserved Tyr residues, two have been shown to play a role in substrate binding, and there is a single conserved Trp^{141}. Other conserved residues whose potential functions remain to be explored include Thr^{304}, two additional acidic residues (Asp^{287}, Asp^{185}), and a single conserved basic residue (Arg^{256}). These conserved residues may be involved in the copper-mediated interaction of these monooxygenases with molecular oxygen.

This proposed model suggests that the two domains could be held together by a hinge akin to the flexible hinges found in mitochondrial creatine kinase (Gross et al., 1996) and porcine pepsin (Abad-Zapatero et al., 1990), where the substrate could come into the active site cavity formed between the two domains. The spectroscopic analysis of oxidized and reduced bifunctional PAM has shown that reduction of the coppers is accompanied by large structural changes in the active site; in contrast, binding of peptidylglycine substrate is not accompanied by major structural changes (Boswell et al., 1996). The two-domain model we have proposed accommodates a structural change of this nature. Ultimately the model will be confirmed by solution of the crystal structure of this protein (Prigge et al., 1997). This proposed model allows us to further our understanding of the PHM reaction and homologous reactions so that the enzyme activity can be manipulated in vivo.

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REFERENCES

Abad-Zapatero, C., Rydel, T. J., & Erickson, J. (1990) *Proteins: Struct., Funct., Genet. 8*, 62.

Bennett, H. P. J. (1991) in *High-performance Liquid Chromatography of Peptides and Proteins—Separation, Analysis, and Conformation* (Mant, C. T., & Hodges, R. S., Eds.) pp 319−325, CRC Press, Inc., Boca Raton, FL.
Blumberg, W. E., Desai, P. R., Powers, L., Freedman, J. H., & Villafranca, J. J. (1989) *J. Biol. Chem. 264*, 6029.
Boswell, J. S., Reedy, B. J., Kulathila, R., Merkler, D. J., & Blackburn, N. J. (1996) *Biochemistry 35*, 12241.
Bradbury, A. F., & Smyth, D. G. (1991) *Trends Biochem. Sci. 16*, 112.
Bradbury, A. F., Mistry, J., Roos, B. A., & Smyth, D. G. (1990) *Eur. J. Biochem. 189*, 363.
Casara, P., Ganzhorn, A., & Danzin, C. (1996) *Bioorg. Med. Chem. Lett. 6*, 393.
DeWolf, W. E. J., Carr, S. A., Varrichio, A., Goodhart, P. J., Mentzer, M. A., Roberts, G. D., Southan, C., Dolle, R. E., & Kruse, L. I. (1988) *Biochemistry 27*, 9093.
Eipper, B. A., Mains, R. E., & Glembotski, C. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5144.
Eipper, B. A., Perkins, S. N., Husten, E. J., Johnson, R. C., Keutmann, H. T., & Mains, R. E. (1991) *J. Biol. Chem. 266*, 7827.
Eipper, B. A., Green, C. B., Campbell, T. A., Stoffers, D. A., Keutmann, H. T., Mains, R. E., & Ouafik, L. (1992a) *J. Biol. Chem. 267*, 4008.
Eipper, B. A., Stoffers, D. A., & Mains, R. E. (1992b) *Annu. Rev. Neurosci. 15*, 57.
Eipper, B. A., Quon, A. S., Mains, R. E., Boswell, J. S., & Blackburn, N. J. (1995) *Biochemistry 34*, 2857.
Erion, M. D., Tan, J., Wong, M., & Jeng, A. Y. (1994) *J. Med. Chem. 37*, 4430.
Farrington, G. K., Kumar, A., & Villafranca, J. J. (1990) *J. Biol. Chem. 265*, 1036.
Freeman, J. C., Villafranca, J. J., & Merkler, D. J. (1993) *J. Am. Chem. Soc. 115*, 4923.
Gilligan, J. P., Lovato, S. J., Mehta, N. M., Bertelsen, A. H., Jeng, A. Y., & Tamburini, P. P. (1989) *Endocrinology 124*, 2729.
Gross, M., Wyss, M., Furter-Graves, E. M., Wallimann, T., & Furter, R. (1996) *Protein Sci. 5*, 320.
Horton, R. M., Cai, Z. L., Ho, S. N., & Pease, L. R. (1990) *BioTechniques 8*, 528.
Husten, E. J., & Eipper, B. A. (1991) *J. Biol. Chem. 266*, 17004.
Husten, E. J., Tausk, F. A., Keutmann, H. T., & Eipper, B. A. (1993) *J. Biol. Chem. 268*, 9709.
Iwasaki, Y., Shimoi, H., Saiki, H., & Nishikawa, Y. (1993) *Eur. J. Biochem. 214*, 811.
Jordan, M., Schallhorn, A., & Wurm, F. M. (1996) *Nucleic Acids Res. 24*, 596.
Katopodis, A. G., & May, S. W. (1990a) *Biochemistry 29*, 4541.
Katopodis, A. G., & May, S. W. (1990b) *Biochemistry 29*, 6115.
Kizer, J. S., Bateman, R. C., Jr., Miller, C. R., Humm, J., Busby, W. H., Jr., & Youngblood, W. W. (1986) *Endocrinology 118*, 2262.
Klinge, M., Cheng, H., & Zabriskie, T. M. (1994) *J. Chem. Soc., Chem. Commun.*, 1379.
Kolhekar, A. S., Roberts, M. S., Jiang, N., Mains, R. E., Eipper, B. A., & Taghert, P. H. (1997) *J. Neurosci. 17*, 1363.
Lee, T. D., & Shively, J. E. (1990) *Methods Enzymol. 193*, 361.
Merkler, D. J., Kulathila, R., Consalvo, A. P., Young, S. D., & Ash, D. E. (1992) *Biochemistry 31*, 7282.
Merkler, D. J., Kulathila, R., & Ash, D. E. (1995a) *Arch. Biochem. Biophys. 317*, 93.
Merkler, D. J., Kulathila, R., Francisco, W. A., Ash, D. E., & Bell, J. (1995b) *FEBS Lett. 366*, 165.
Milgram, S. L., Johnson, R. C., & Mains, R. E. (1992) *J. Cell Biol. 117*, 717.
Monastirioti, M., Linn, C. E., Jr., & White, K. (1996) *J. Neurosci. 16*, 3900.
Mounier, C. E., Shi, J., Sirimanne, S. R., Chen, B. H., Moore, A. B., Gill-Woznichak, M. M., Ping, D., & May, S. W. (1997) *J. Biol. Chem. 272*, 5016.
Ouafik, L.', May, V., Keutmann, H. T., & Eipper, B. A. (1989) *J. Biol. Chem. 264*, 5839.
Pedersen, J. Z., & Finazzi-Agro, A. (1993) *FEBS Lett. 325*, 53.
Prigge, S. T., Kolhekar, A. S., Eipper, B. A., & Amzel, L. M. (1997) *Biophys. J. 72*, A223 (Abstract).
Ramer, S. E., Cheng, H., Palcic, M. M., & Vederas, J. C. (1988) *J. Am. Chem. Soc. 110*, 8526.
Reedy, B. J., Murthy, N., Karlin, K. D., & Blackburn, N. J. (1995) *J. Am. Chem. Soc. 117*, 9826.
Rhodes, C. H., & Honsinger, C. (1993) *Ann. N.Y. Acad. Sci. 689*, 663.
Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49.
Robertson, J. G., Adams, G. W., Medzihradszky, K. F., Burlingame, A. L., & Villafranca, J. J. (1994) *Biochemistry 33*, 11563.
Smith, D. L., & Zhou, Z. (1990) *Methods Enzymol. 193*, 374.
Southan, C., & Kruse, L. I. (1989) *FEBS Lett. 255*, 116.
Stoffers, D. A., Green, C. B., & Eipper, B. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 735.
Takahashi, K., Okamoto, H., Seino, H., & Noguchi, M. (1990) *Biochem. Biophys. Res. Commun. 169*, 524.
Tausk, F. A., Milgram, S. L., Mains, R. E., & Eipper, B. A. (1992) *Mol. Endocrinol. 6*, 2185.
Tian, G., Berry, J. A., & Klinman, J. P. (1994) *Biochemistry 33*, 226.
Wernicke, D., & Will, H. (1992) *Anal. Biochem. 203*, 146.
Yonekura, H., Anzai, T., Kato, I., Furuya, Y., Shizuta, S., Takasawa, S., & Okamoto, H. (1996) *Biochem. Biophys. Res. Commun. 218*, 495.
Young, S. D., & Tamburini, P. P. (1989) *J. Am. Chem. Soc. 111*, 1933.
Zabriskie, T. M., Cheng, H., & Vederas, J. C. (1992) *J. Am. Chem. Soc. 114*, 2270.

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