Peptidylglycine-α-Hydroxylating Monooxygenase Generates Two Hydroxylated Products from Its Mechanism-Based Suicide Substrate, 4-Phenyl-3-butenoic Acid†

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ABSTRACT: The bifunctional enzyme peptidylglycine-α-amidating monooxygenase mediates the conversion of C-terminal glycine-extended peptides to their active α-amidated products. Peptidylglycine-α-hydroxylating monooxygenase (PHM, EC 1.14.17.3) catalyzes the first reaction in this two-step process. The olefinic compound 4-phenyl-3-butenoic acid (PBA) is the most potent irreversible, mechanism-based PHM inactivator known. While the details of the inhibitory action of PBA on PHM remain undefined, covalent modification of the protein has been proposed as the underlying mechanism. We report here that, in the process of inactivating PHM, PBA itself serves as a substrate without covalently labeling the enzyme. Approximately 100 molecules of PBA are metabolized per molecule of PHM inactivated, under saturating conditions. The metabolism of PBA by PHM generates two hydroxylated products, 2-hydroxy-4-phenyl-3-butenoic acid and its allylic isomer, 4-hydroxy-4-phenyl-2-butenoic acid. While one enantiomer for each product is significantly favored in the reaction, both are produced. From these observations, we conclude that hydroxylated PBA products are formed by a delocalized free radical mechanism and that the lack of absolute stereospecificity indicates significant freedom of movement within the catalytic site. The ability of PHM to metabolize PBA suggests that the physiological functions of PHM may include the hydroxylation of substrates other than those containing terminal glycines.

Intercellular peptide messengers regulate physiological mechanisms essential for life. More than half of all known neuroendocrine peptides are α-amidated at their carboxy-termini, and in most cases this structural feature is requisite for receptor recognition and signal transduction (*1*−*3*). α-Amidation is catalyzed by peptidylglycine-α-amidating monooxygenase (PAM),[1] which constitutes the only known mechanism for generating α-amidated peptides in vivo. PAM is a bifunctional enzyme consisting of peptidylglycine-α-hydroxylating monooxygenase (PHM, EC 1.14.17.3) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL, EC 4.3.2.5). The hydroxylase and lyase domains of PAM sequentially catalyze the two steps required for converting glycine-extended peptide precursors to active peptide amides (*1*−*3*). PHM is rate-limiting in this sequence and thus represents a key control point in the bioactivation of peptide messengers. The PHM and PAL domains of PAM may be separated with full retention of their respective enzymatic activities (*4*−*6*). This feature has facilitated the study of each catalytic domain. To date, attention has focused on PHM because of its regulatory role in controlling α-amidation and its homology to the norepinephrine synthesizing enzyme, dopamine β-monooxygenase (DBM, EC 1.14.17.1) (*7*,*8*).

The crystallographic structure of PHM has provided considerable insight into the mechanism by which PHM hydroxylates its substrates (*9*). PHM is composed of 2 domains that are approximately equal in size (∼150 residues). Each domain coordinates one copper atom on opposite sides of a solvent-accessible catalytic cleft. This open coordination positions the two copper atoms (CuA and CuB) 11 Å apart and, thus, precludes the formation of a typical binuclear center in which oxygen bridges the two metal ions. Substrate-mediated electron transfer between the metal ions (*10*) and channeling of superoxide (*11*) have been proposed to play a

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[1] Abbreviations: PAM, peptidylglycine-α-amidating monooxygenase; PHM, peptidylglycine-α-hydroxylating monooxygenase; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; DBM, dopamine β-monooxygenase; PBA, 4-phenyl-3-butenoic acid; 2-OH-PBA, 2-hydroxy-4-phenyl-3-butenoic acid; 4-OH-PBA, 4-hydroxy-4-phenyl-2-butenoic acid; NMR, nuclear magnetic resonance; LC/MS, liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; CID, collision-induced dissociation; MES, 2-(*N*-morpholino)-ethanesulfonic acid; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, poylacrylamide gel electrophoresis.

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role in the reaction. The copper atoms are reduced in two one-electron transfers by ascorbate, generating two semidehydroascorbates (*12*). The two coppers atoms, however, are not functionally equivalent (*13*), and only reduced CuB serves as the coordination site for molecular oxygen (*14*). The primary driving force for positioning peptide substrates within the active site is hydrogen bonding (*9*). These interactions place the α-carbon of the terminal glycine adjacent to the activated oxygen bound at CuB. In the ternary complex of reduced enzyme, O_2, and substrate, the copper atoms are reoxidized by transferring one electron each to molecular oxygen. The hydroxylation reaction proceeds stereospecifically with the abstraction of the *pro-S* α-hydrogen of the terminal glycine residue and subsequent hydroxylation with retention of configuration (*15*, *16*). In the process, one atom from molecular oxygen is incorporated into the hydroxyl moiety (*14*, *17*), and the other forms water by combining with the hydrogen atom abstracted from substrate and a solvent proton.

The open conformation of the catalytic site is presumably needed to accommodate a wide variety of substrates as well as reductant. PHM readily hydroxylates peptides in which the C-terminal glycine is preceded by any of the naturally occurring L-amino acids and accommodates chain lengths ranging from 3 to 66 residues (*2*, *18*). The structural freedom within the catalytic cleft may also explain why nonpeptide compounds can serve as substrates or inhibitors (*19*). Of this group, the most potent irreversible mechanism-based inhibitor of PHM is the olefinic substrate analogue 4-phenyl-3-butenoic acid (PBA) (*20*). The use of PBA as a PHM inhibitor has become an important tool for investigating PAM action and is now considered a benchmark for characterizing PAM activity both in vitro (*21*−*23*) and in vivo (*24*−*26*).

The present report arose from attempts to identify potential sites at which the structure of PHM might be modified for physiologic regulation. Recent findings indicate that covalent modification mediates a marked increase in the enzyme's V_{max} in response to in vivo disulfiram (Antabuse) administration (*27*). Disulfiram impairs α-amidated peptide synthesis by limiting copper availability (*28*), and the increased V_{max} is thought to occur as a compensatory response to the deficit. Because PBA had been reported to irreversibly inactivate PHM through covalent modification (*29*), we intended to use it as a tool to identify sites where PHM might be modified physiologically in response to disulfiram treatment. We found, instead, that PBA did not form a covalent adduct with PHM protein but served as an effective substrate while concurrently inactivating the enzyme. PHM produced two distinct hydroxylated products from PBA, 2-hydroxy-4-phenyl-3-butenoic acid and 4-hydroxy-4-phenyl-2-butenoic acid, with a partition ratio of approximately 100 molecules of PBA metabolized per molecule of PHM inactivated. The ability of PHM to catalyze the dual hydroxylation of PBA suggests considerable freedom of movement within the binding pocket and further raises the possibility that the normal functions of PHM may include the hydroxylation of substrates other than glycine-extended compounds.

MATERIALS AND METHODS

Materials and Radiochemicals. 4-Phenyl-3-butenoic acid (PBA, *trans*-styrylacetic acid, 96%) was purchased from Aldrich Chemical Co., melting point (mp) 84−86 °C, and analyzed by NMR: ^{1}H NMR 200 MHz ($CHCl_3$-*d*) δ 7.31 (m, 5H), 6.52 (dd, 1H, J_{34} = 16.0 Hz), 6.27 (m, 1H), 3.30 (dd, 2H, J_{23} = 6.0, J_{24} = 2.2 Hz). None of the cis isomer was detected in the NMR spectrum. This compound was radiolabeled by tritium exchange (10.7 Ci/mmol) by New England Nuclear Life Sciences Products Custom Services and purified by reverse phase HPLC before use.

Synthesis of (±)-2-Hydroxy-4-phenyl-3-butenoic Acid. Ten milligrams of crude 2-oxo-4-phenyl-3-butenoic acid (Aldrich Chemical Co., Rare Chemicals) was combined with 10 mg of sodium borohydride (Aldrich Chemical Co.) in methanol and allowed to stand for 1 h at room temperature. The solution was then acidified to pH 5 with acetic acid, extracted with ether, and evaporated. The resulting product, approximately 5 mg of an oil, produced several $(M-H)^-$ ions in negative ion electrospray mass analysis (described below), including one at *m*/*z* 177. This ion provided the collision-induced dissociation (CID) mass spectrum shown in Figure 7B. Further purification by HPLC (described below) yielded a product which showed only a single ion at *m*/*z* 177 with the same CID spectrum as that observed in the crude mixture. Its identity was confirmed by comparison of its ^{1}H NMR 200 MHz ($CHCl_3$-*d*) spectrum with that of a sample of the dextrorotatory (*R*)-2-hydroxy-4-phenyl-3-butenoic acid kindly provided by Dr. A. Matsuyama of Daicel Chemical Industries, Ltd., Tsukuba-City, Ibaraki, Japan (*30*). ^{1}H NMR 200 MHz (MeOH-*d*) δ 7.30 (m, 5H), 6.83 (dd, 1H, J_{23} = 16.0 Hz, J_{13} = 2.0 Hz), 6.26 (dd, 1H, J_{12} = 6.0 Hz), 4.93 (dd, 1H).

Synthesis of (±)-4-Hydroxy-4-phenyl-2-butenoic Acid. This compound was prepared from 360 mg of 3-benzoylacrylic acid (Lancaster Synthesis) by reduction with 130 mg of $NaBH_4$ in 50 mL of water and 10 mL of ether to enhance solubility of the acid. After 1 h, the solution was acidified to pH 5 with acetic acid, filtered, and then extracted with ether. Evaporation of the ether extract yielded 227 mg of an oil which subsequently produced a crystalline material with a mp of 75−83 °C. Recrystallization from $CHCl_3$−isooctane (1:1, v/v) raised the mp to 92−94 °C, literature mp (*31*): 94 °C. ^{1}H NMR 200 MHz (MeOH-*d*) δ 7.37 (s, 5H), 6.99 (dd, 1H, J_{23} = 15.6 Hz), 5.35 (dd, 1H, J_{24} = 1.5, J_{34} = 5.1 Hz); literature values (*31*): ^{1}H NMR 80 MHz (DMSO-d_6) δ 7.38 (s, 5H), 6.87 (dd, 1H), 6.04 (dd, 1H, J_{23} = 15 Hz), 5.30 (dd, 1H, J_{24} = 2.4 Hz, J_{34} = 4.8 Hz), 8.1 (br s, exch. D_2O, OH).

Action of m-Chloroperoxybenzoic Acid on 4-Phenyl-3-butenoic Acid. A solution of 126 mg of PBA was combined with 185 mg of *m*-chloroperoxybenzoic acid (Aldrich Chemical Co.) in $CHCl_3$, stirred overnight, and then dried by evaporation. The resulting residue was taken up in 30 mL of hot water, filtered, and concentrated by evaporation to approximately 10 mL. Upon cooling, 80 mg of hexagonal crystals of *trans*-3-hydroxy-4-phenylbutyrolactone precipitated, mp 80−92 °C. Recrystallization from water raised the mp to 89−92 °C; negative ion electrospray showed an $(M-H)^-$ ion at *m*/*z* 177. ^{1}H NMR 200 MHz (MeOH-*d*) δ 7.36 (s, 5H), 5.39 (d, 1H, J_{34} = 3 Hz), 4.48 (m, 1H), 2.85 (dd, 1H, $J_{22'}$ = 18 Hz, $J_{2'3}$ = 6 Hz), 2.58 (dd, 1H, J_{23} = 4.4 Hz). A minor isomer, presumably formed by isomerization at the benzyl carbonium ion during lactonization, was resolved (R_f = 0.54) from the major isomer (R_f = 0.68) by preparative

TLC on 100μ silica G plates (Aldrich Chemical Co.) using a solvent system of 0.5% acetic acid in 1:1 (v/v) benzene−ethyl acetate. Negative ion electrospray showed an $(M-H)^-$ ion at *m/z* 177. ^{1}H NMR 200 MHz (MeOH-*d*) δ 7.39 (s, 5H), 5.45 (d, 1H, $J_{34} = 3.5$ Hz), 4.58 (m, 1H), 3.07 (dd, 1H, $J_{22'} = 17.5$ Hz, $J_{23} = 5.3$ Hz), 2.47 (d, 1H, $J_{2'3} =$ Hz). For this compound, the cis (nearly eclipsed) orientation of the protons accounts for the lack of coupling of one of the C-2 and C-3 protons.

Preparation of Recombinant PHM Catalytic Core (PHMcc). Recombinant PHM protein (amino acids 42−356 of rat PAM) was expressed in Chinese hamster ovary cells (*32*) and purified as described previously (*33*). PHM protein recovered from 1 L of culture medium was 2.25 mg at a purity of >98% as assessed by silver-stained SDS−polyacrylamide gel electrophoresis. PHM activity was assayed as described (*33*) using [^{125}I]iodo-α-N-Ac-Tyr-Val-Gly substrate. Protein concentrations were estimated by the method of Lowry et al. (*34*) using bovine serum albumin as the standard.

PHM Inactivation and Generation of PBA Products. PBA-mediated inactivation of PHMcc and generation of PBA metabolites were performed in a total volume of 100 μL of 150 mM MES, pH 5.0, containing 2 μM $CuSO_4$, 1 mM ascorbic acid, and 300 μg/mL catalase (Sigma Chemical Co). For specific experiments, the reaction composition was altered as indicated in tables and figure legends. PBA was added from concentrated stock solutions prepared in 10% ethanol so that the final ethanol concentration did not exceed 1%. For reactions in which PBA was omitted, an appropriate volume of 10% ethanol was substituted. PHMcc was diluted in PHM sample diluent [10 mM Tris, pH 7.0, containing 1% Triton X-100 (Surfact-Amps X-100, Pierce Chemical Co.) and 0.2 mg/mL bovine serum albumin] and added so that the final concentration of diluent was 5−20%. For reactions in which PHMcc was omitted, an appropriate volume of PHM sample diluent was substituted. The presence of PHM sample diluent and catalase enhanced the stability of PHM activity. Incubations were carried out at 37 °C for 10−60 min as indicated in the figure legends.

Reverse-Phase HPLC. Chromatography was performed using a 4.6 × 250 mm Vydac C_4 column (The Nest Group Inc.) on a series 1100 HPLC system equipped with ChemStation software, diode array detector, and thermostated column compartment (Hewlett-Packard). The system was operated at a flow rate of 1 mL/min, and a column temperature of 40 °C. The column was equilibrated with 97.5% solvent A (0.1% TFA and 2.5% acetonitrile in water) and 2.5% solvent B (0.08% TFA in acetonitrile). Following sample injection, initial conditions were maintained for 5 min, after which a linear gradient to 42.5% solvent B was developed over the next 10 min. Isocratic conditions were maintained at 42.5% solvent B for 3 min followed by an increase to 92.5% solvent B over 0.5 min. Elution profiles were monitored at 280, 240, and 214 nm, and peaks were collected manually.

Chiral Chromatography. Separation of stereoisomers was performed by HPLC. Enantiomers of 4-OH-PBA were separated on a 250 × 2.l mm Chirose C1 column (Phenomenex) run under isocratic conditions (15% acetonitrile in water, 0.1% TFA) at a flow rate of 0.25 mL/min and a temperature of 20 °C. Enantiomers of 2-OH-PBA were separated on a 50 × 4.0 mm Chirex COO5 column (Phenomenex) run under isocratic conditions (25 mM ammonium acetate in methanol) at a flow rate of 0.5 mL/min and a temperature of 30 °C.

Mass Spectrometry. For data presented in Figure 7, analyses were carried out using a Finnigan TSQ 700 mass spectrometer (Finnigan MAT) with a modified ion source (*35*). Sample was delivered at a rate of 100 nL/min into a spray voltage of 2.5 kV; the capillary temperature was maintained at 200 °C. CID mass spectra were obtained at a collision gas (argon) pressure of 0.5 mTorr and a collision offset voltage of 30 eV in the negative ion mode. Alternatively, for data presented in Figure 8, analyses were performed using a Hewlett-Packard series 1100 integrated LC-electrospray mass spectrometer operated in the negative ion mode. Reverse-phase liquid chromatography was performed as described above except that 1% and 0.8% acetic acid was substituted for TFA in buffers A and B, respectively.

Table 1: Metabolism of [^{3}H]PBA by PHMcc

reaction condition[a]	radioactivity (cpm × 10^{-3}) PBA	PHMcc
minus PHM	866	3
inactive PHM[b]	936	8
active PHM	83	8
active PHM + nonradioactive PBA[c]	340	10

[a] [^{3}H]PBA (10.7 Ci/mmol) was incubated with and without PHMcc as indicated. Reactions were carried out in a total volume of 100 μL of 150 mM MES, pH 5.0, containing 2 μM Cu^{2+}, 1 mM ascorbate, and 200 μg/mL catalase at 37 °C for 60 min. Reaction mixtures were separated by HPLC, and fractions corresponding to the elution times of PBA and PHMcc were collected. Radioactivity of the fractions was measured by scintillation counting. [b] PHMcc was inactivated by acid denaturation (0.1% TFA). [c] 500 μM radioinert competing PBA included in the reaction mixture.

RESULTS

Detection of PBA Reaction Products. Repeated attempts to covalently label PHMcc with [^{3}H]PBA resulted in a dramatic consumption of PBA under conditions that completely inactivated the enzyme; however, no specific incorporation of radioactivity into PHM protein was evident (Table 1). The small amount of radioactivity (<1%) that eluted with PHM protein on HPLC was determined to be nonspecific. This radioactivity was not decreased by an excess of nonradioactive competing PBA or when inactivated PHM was substituted for active enzyme. On the other hand, reaction of [^{3}H]PBA with active enzyme reduced the radioactivity in the PBA peak to less than 10% of control. This effect was significantly attenuated by the addition of unlabeled PBA to the reaction. In the absence of PHMcc or with PHMcc previously inactivated by denaturation, >99% of the radioactivity was recovered in the PBA peak. It was evident, therefore, that PBA was being metabolized while inactivating PHM without forming a covalent adduct with the protein.

Further analysis of the [^{3}H]PBA/PHMcc reaction mixtures by reverse-phase HPLC revealed the generation of three radioactive products (Figure 1). The first (peak 1) eluted with ascorbic acid and buffer salts at the solvent front (3−4 min), and the other two eluted at 12.5 and 14.0 min (peaks 2 and

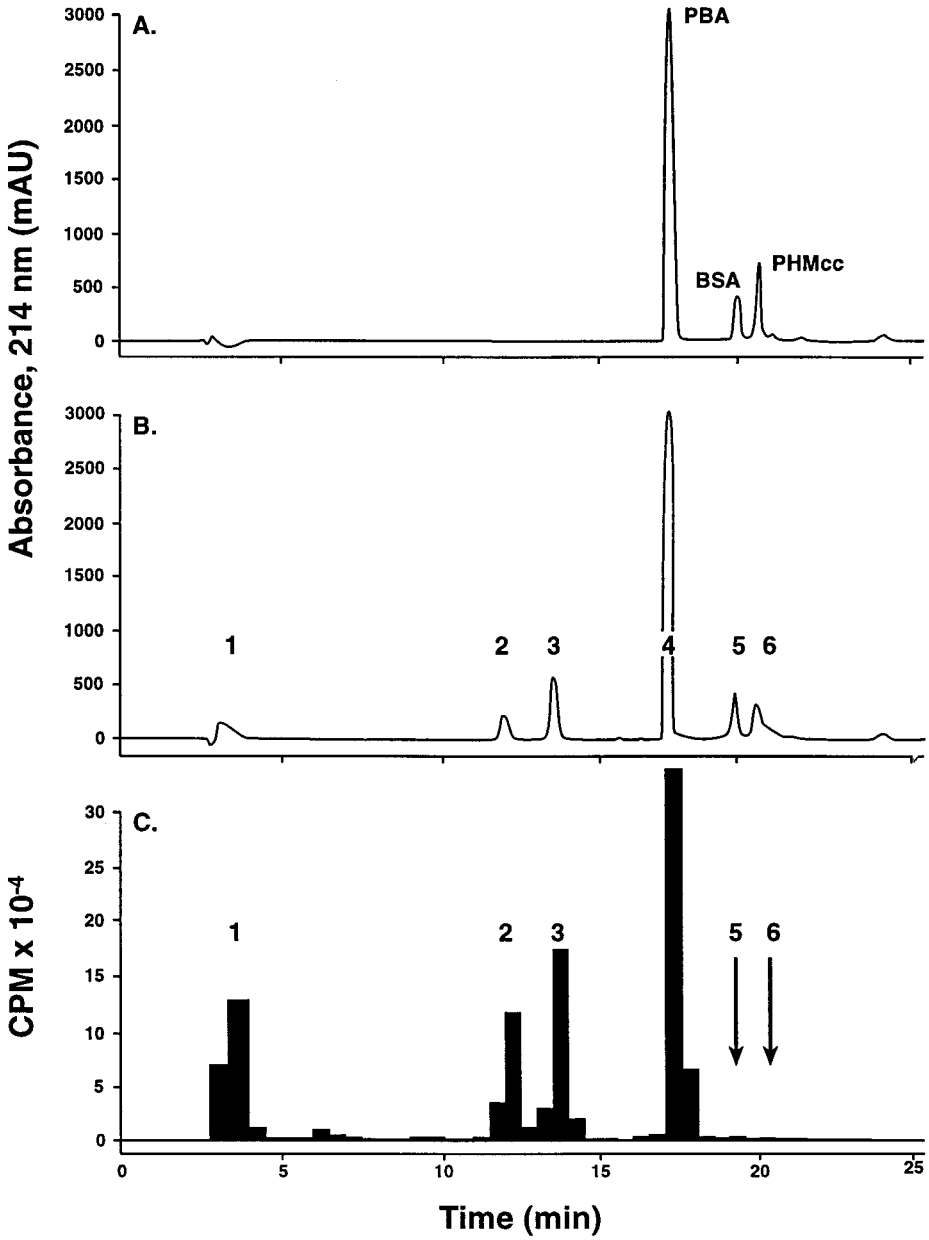

FIGURE 1: HPLC profiles for PBA reaction mixtures. PHMcc was incubated with 500 μM PBA (1.1×10^8 cpm of [^{3}H]PBA) for 60 min, and reaction components were separated by HPLC. Panel A, incubation without ascorbic acid (no reaction); panel B, incubation with ascorbic acid (complete reaction); panel C, radioactivity in 0.5 mL fractions collected across the profile shown in panel B. Peaks are numbered by order of elution, and those corresponding to PBA, bovine serum albumin (BSA), and PHMcc are indicated in panel A.

3, respectively); unmetabolized PBA substrate (peak 4) eluted at 17.5 min (panel C). The retention times for the radioactivity in peaks 2 and 3 coincided precisely with distinct peaks of UV absorbance visualized in reactions containing high concentrations (500 μM) of nonradioactive PBA (panel B). Peaks of UV absorbance representing BSA, a constituent of sample diluent (peak 5), and PHMcc (peak 6) (panels A and B) were not associated with appreciable radioactivity under conditions where [^{3}H]PBA was metabolized to its three respective products (peaks 1, 2, and 3; panel C). Importantly, the total radioactivity found in peaks 1, 2, and 3 fully accounted for the loss of radioactivity from the PBA peak. Furthermore, because sufficient quantities of peak 2 and 3 reaction products were produced for detection by UV absorption, nonradioactive PBA could be used for subsequent experiments.

Characterization of PHM Inactivation and PBA Product Generation. The data presented in Table 2 show that the generation of PBA products and the inactivation of PHM protein are coordinate events requiring the same cofactors necessary for the enzymatic hydroxylation of peptidylglycine substrates. In the presence of optimal concentrations of copper and ascorbate, incubation of PHMcc with PBA resulted in the generation of PBA products depicted in Figure 1 and complete inactivation of the enzyme. Dopamine could replace ascorbate as reductant with equally robust product formation and potent inactivation (data not shown). In contrast, when incubations were performed in the absence of reductant, there was no evidence of PBA product formation, and PHM activity was largely preserved (12% inactivation). Without exogenously added copper, 50% of PHM activity was retained, and PBA product generation was

Table 2: Cofactor Dependence of PBA-Mediated PHM Inactivation and PBA Metabolite Production

reaction variable				response (% of maximum)	
PBA (100 μM)	ascorbate (1 mM)	Cu^{2+} (2 μM)	EDTA (30 μM)	PHM inactivation[a]	PBA metabolism[b]
−	+	+	−	0	0
+	+	+	−	99	100
+	−	+	−	12	0
+	+	−	−	50	24
+	+	+	+	12	0

[a] Incubations with the indicated variables were carried out at 37 °C in 150 mM MES, pH 5.0, containing 12.3 pmol of PHMcc in PHM sample diluent and 300 μg/mL catalase (total volume, 100 μL). After 10 min, an aliquot (5 μL) was taken and diluted with ice-cold PHM sample diluent and assayed for PHM activity in duplicate. [b] Following removal of the aliquot (5 μL) for PHM activity, reactions were stopped by the addition of 3.5 μL of 10% TFA/acetonitrile (1:2.5, v/v) and products separated by reverse-phase HPLC. The relative amount of PBA metabolism was measured by summing the peak heights for PBA reaction product peaks 2 and 3.

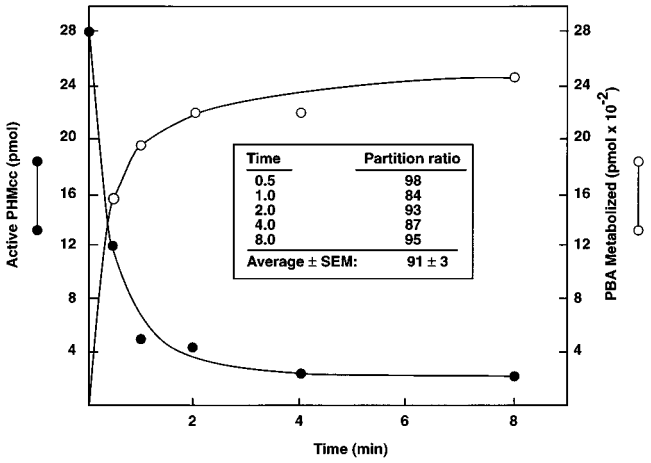

FIGURE 2: Time course for PHM inactivation and PBA metabolism. PHMcc (28 pmol) was incubated at 37 °C with 100 μM PBA in 150 mM MES, pH 5.0, containing 2 μM $CuSO_4$, 0.2 mg/mL ascorbic acid, and 1.5 mg/mL catalase (total volume 100 μL). Aliquots were removed at the times indicated, diluted 1:100 in PHM diluent, and snap-frozen on dry ice for subsequent determination of PHM activity. PHM activity data points represent the average of assays performed in duplicate and are expressed as picomoles of active PHM (●). Reactions for determining PBA metabolism were stopped by acidification and fractionated by HPLC. PBA consumption (○) was calculated from changes in PBA peak heights. The partition ratio (picomoles of PBA metabolized per picomoles of PHM inactivated) at each time point is shown in the inset. The mean of the data points and the standard error (SEM) are indicated. The data shown are representative of three independent experiments.

only 24% of that observed under optimal conditions. When EDTA was used to chelate any copper that copurified with PHMcc, no PBA products were generated, and PHMcc retained 88% of its activity. Additionally, a 20-fold molar excess of peptide substrate over PBA prevented the formation of PBA products and protected PHMcc from inactivation (data not shown), presumably by preventing access of PBA to the catalytic site. Importantly, neither peak 2 nor peak 3 reaction products inactivated PHMcc when incubated with the enzyme under conditions and concentrations used for PBA inactivation. Thus, both the generation of PBA products and the inactivation of PHM are the result of active catalysis.

Figure 2 shows a time course for PHM inactivation and PBA metabolism. The ratios of picomoles of PBA consumed per picomoles of PHMcc inactivated at each time point are shown in the inset. The calculated mean for the partition ratio was 91 ± 3 pmol of PBA metabolized/pmol of PHMcc inactivated. Two additional time course experiments conducted in a similar manner resulted in partition ratios of 103 ± 7 and 99 ± 8 pmol/pmol.

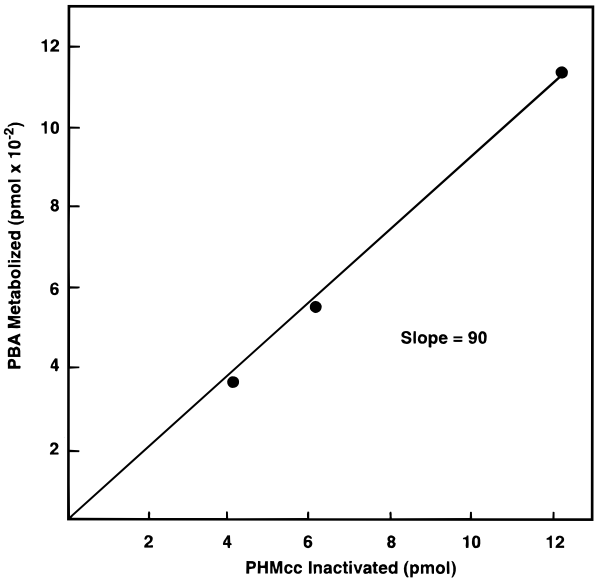

FIGURE 3: Partition between PBA metabolism and PHM inactivation. PHMcc (4.1, 6.15, or 12.3 pmol) was incubated with 200 μM PBA at 37 °C for 10 min as described in Figure 2. Reactions were acidified and fractionated by HPLC. Under these conditions, PBA inactivation of PHM is complete. PBA consumption was calculated from the changes in PBA peak heights compared to the control incubation that did not contain PHMcc. The slope of the line is the partition ratio.

A partition ratio was also obtained by incubating increasing amounts of PHMcc with a constant amount of PBA (Figure 3). The results of these studies demonstrated a linear relationship with a partition ratio of 90 pmol of PBA consumed/pmol of PHM inactivated. It is important to point out that these experiments for determining partition ratios were conducted at saturating concentrations of PBA. At lower concentrations, the partition ratio decreased (data not shown). Under conditions where the amount of PHMcc was not initially limiting, PBA products were generated linearly up to 10 μM PBA (Figure 4). On the basis of this and similar experiments, the Michaelis constant (K_m) for PBA was estimated to be 22 μM. This value, however, represents an approximation because PHM is being inactivated throughout the course of the reaction. Pulse−chase experiments were performed to ascertain if the PBA products were generated sequentially, one from another. When [^{3}H]PBA reactions were chased with nonradioactive PBA, there was no detectable shift in the amounts of radioactivity associated with any of the products upon further incubation (data not shown). The continued generation of UV-detectable PBA products during the chase period confirmed that these data were not the result of complete inactivation of PHM and demonstrated that each reaction product was generated independently.

Characterization and Identification of PBA Products. When peak 1 (see Figure 1) was diluted in water and distilled, the radioactivity was recovered in direct proportion to the volume of water collected (data not shown). Peak 1 radioactivity was immiscible in hexane and only slightly

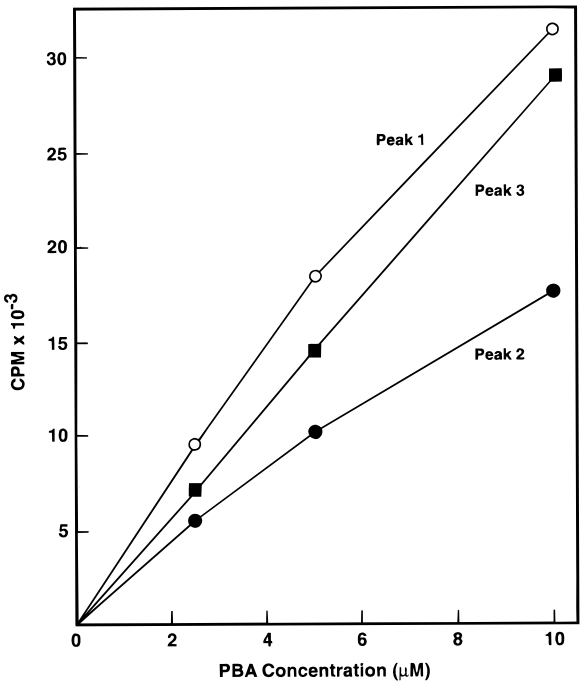

FIGURE 4: Dependence of PBA product generation on PBA concentration. PHMcc (50 pmol) was incubated for 15 min at 37 °C with 2.5, 5.0, and 10 μM [^{3}H]PBA (specific activity = 13.0 × 10^7 cpm/μmol) as described in Figure 2. Reactions were stopped by acidification and fractionated by HPLC. Peaks 1 (○), 2 (●), and 3 (■) (see Figure 1) were collected, and radioactivity was determined by scintillation counting.

soluble in ethyl acetate and dichloromethane. The modest amount of radioactivity that was extracted into ethyl acetate, however, could be removed by the addition of desiccant (Molecular Sieve). Taken collectively, these data strongly indicate that the peak 1 PBA reaction product is water. In contrast to peak 1, peaks 2 and 3 were retained on reverse-phase HPLC; since they eluted prior to PBA, they appeared to be more polar compounds. The radioactivity from both peaks was miscible in ethyl acetate at low pH (~3.0) but not high pH (~8.5), suggesting the presence of an ionizable group such as a carboxyl; neither peak was miscible in hexane at either acidic or basic pH.

UV spectra for peaks 2 and 3 were very different and are shown in Figure 5. The spectrum for peak 3 exhibited two maxima (207 and 250 nm) and was similar but not identical to that of PBA (maxima at 207 and 255 nm). The spectrum for peak 2 displayed only a single maximum in the far-UV at 210 nm. These data suggested that the configuration of the double bond conjugated to the phenyl ring in PBA was preserved in the peak 3 product but was absent in the peak 2 product.

The negative ion collision-induced dissociation (CID) spectrum for HPLC-purified peak 3 product using the Finnigan TSQ-700 instrument is shown in Figure 6A. It is apparent that the $(M-H)^-$ ion at *m*/*z* 177 represents a compound that is 16 atomic mass units greater than the substrate PBA (formula weight: 162), suggesting the addition of oxygen. Decomposition of this anion resulted in losses of CO_2 (*m*/*z* 133) and $CO_2 + H_2O$ (*m*/*z* 115) and produced ions that we formulate to be indenyl and phenyl anions at *m*/*z* 103 and 77, respectively. The mass of the peak 2 product could not be determined by off-line CID because the HPLC-purified material polymerized during concentration by lyophilization (determined by electrospray mass spectrometry, data not shown). However, direct on-line LC/MS (Hewlett-Packard series 1100 instrument) revealed that the mass of the peak 2 product was identical to that of peak 3 (*m*/*z* 177; Figure 7). Furthermore, the skimmer region fragmentation revealed that the loss of CO_2 (*m*/*z* 133) was much less pronounced for this product as compared to peak 3 analyzed under identical conditions, indicating a structural difference between the two compounds near their carboxyl groups.

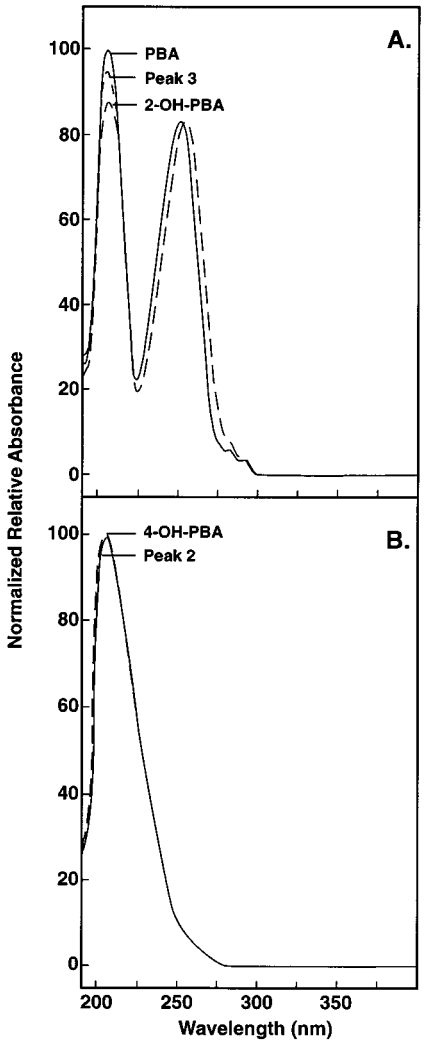

FIGURE 5: UV spectra. Spectra for HPLC peaks were obtained by diode array detection and overlaid for comparison. Panel A, PBA (solid line), peak 3 (dotted line), synthetic 2-OH-PBA (dashed line). Panel B, peak 2 (solid line), synthetic 4-OH-PBA (dashed line).

Collectively, the above data suggested four possible identities for peaks 2 and 3 that would be consistent with the monooxygenase activity of PHM (Figure 8): (a) 3,4-epoxy-4-phenylbutanoic acid, (b) 2,3-epoxy-4-phenylbutanoic acid, (c) 2-hydroxy-4-phenyl-3-butenoic acid (2-OH-PBA), and (d) 4-hydroxy-4-phenyl-2-butenoic acid (4-OH-PBA). Attempts to prepare the first epoxide compound by the action of *m*-chloroperoxybenzoic acid on PBA generated the corresponding *cis*- and *trans*-4-hydroxy-4-phenylbutanoic lactones (see Materials and Methods), which differed considerably in chromatographic and UV spectral properties from those of peaks 2 and 3 (data not shown). The second epoxide was not synthesized because the corresponding 2-butenoic acid was not available. On the other hand, 2-OH-

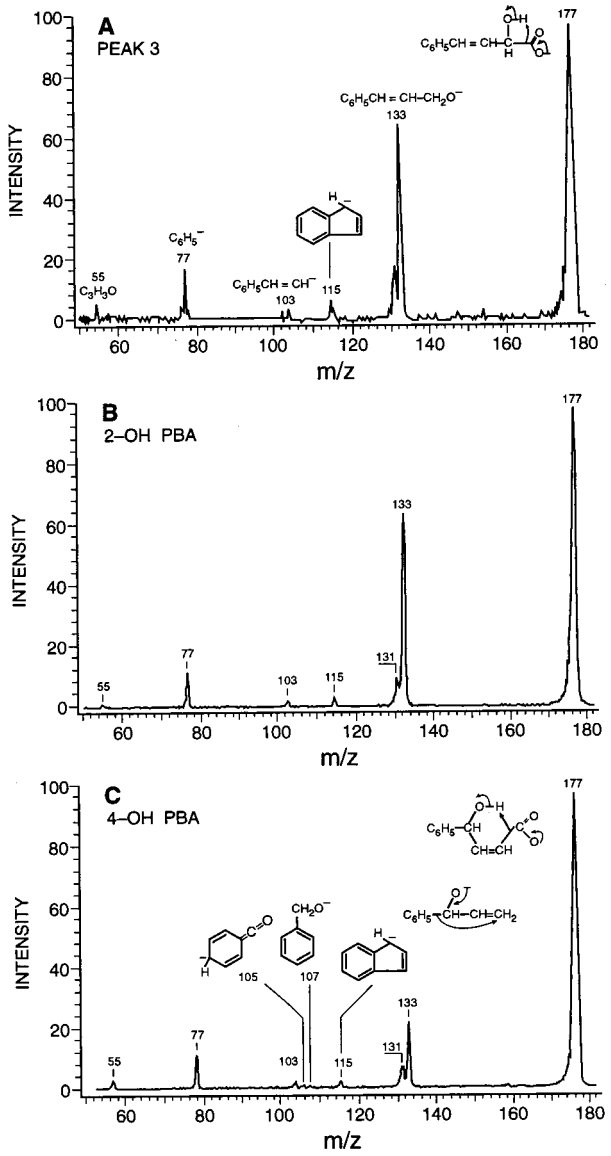

FIGURE 6: Negative ion electrospray CID mass spectra. Panel A, PHM-generated peak 3 isolated by HPLC. Panel B, synthetic (±)-2-OH-PBA. Panel C, synthetic (±)-4-OH-PBA. All spectra were recorded using a Finnigan TSQ 700 mass spectrometer at 0.5 mTorr argon and 30 eV collision energy. The putative daughter ion structures are indicated in panels A and C.

PBA and 4-OH-PBA were easily prepared from their corresponding ketones by reduction with $NaBH_4$ (see Materials and Methods). Analysis of these compounds by reverse-phase HPLC revealed that 4-OH-PBA and 2-OH-PBA migrated with retention times identical to peaks 2 and 3, respectively, and that the UV spectra for the synthetic compounds matched those of the products produced enzymatically (Figure 5). Negative ion CID mass analysis of synthetic 2-OH-PBA produced a fragmentation pattern that was identical to that of peak 3 but was distinct from synthetic 4-OH-PBA (Figure 6). Features of the 4-OH-PBA CID mass spectrum that distinguished it from 2-OH-PBA included the reduced loss of CO_2, as noted with LC/MC (Figure 7), and the generation of benzyloxy and benzoyl anions at *m*/*z* 105 and 107, respectively. These differences were significantly more pronounced at higher collision energies (not shown). The differential loss of CO_2 is consistent with the structures of 4-OH-PBA, an acrylic acid, compared to 2-OH-PBA, an

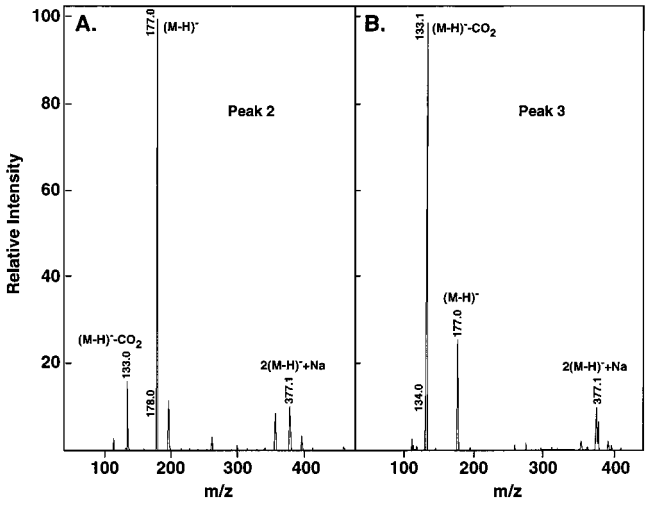

FIGURE 7: Negative ion electrospray LC/MS spectra. PHM-generated PBA reaction products were prepared as described in Figure 2. Reactions were analyzed directly for peaks 2 (panel A) and 3 (panel B) by on-line electrospray mass spectrometry using a Hewlett-Packard series 1100 integrated mass spectrometer in the negative ion mode. Parent ions at *m*/*z* 177 [$(M-H)^-$] and their sodium salt adducts [$2(M-H)^- + Na$] are indicated. The fragmentation ions at *m*/*z* 133 represent the loss of CO_2.

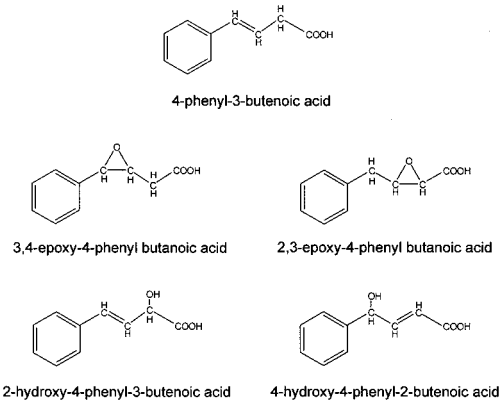

FIGURE 8: Structures for PBA and possible metabolites produced by PHM.

α-hydroxy acid where the transfer of charge from the loss of the carboxyl is better accommodated by the adjacent oxygen. From these data, we conclude that the PBA reaction products of peaks 1, 2, and 3 are water, 4-OH-PBA, and 2-OH-PBA, respectively.

Because the hydroxylation of peptide substrates by PHM proceeds stereospecifically (*16*, *36*), it was of interest to examine the configuration of the PBA reaction products, each of which contains a chiral center at the hydroxylated carbon. Chiral chromatography was performed on peaks 2 and 3 and compared to parallel analyses of racemic mixtures of their corresponding synthetic compounds (Figure 9). Although one stereoisomer was significantly favored over the other, both enantiomers of each product were generated in the reaction. For peak 2, the ratio was 30%:70% (panel C), and for peak 3, the ratio was 80%:20% (panel D). Raising or lowering the oxygen content of the reaction buffer (bubbling O_2 or N_2) did not change these ratios. Use of authentic (*R*)-2-OH-PBA as an internal standard demonstrated that the major stereoisomer of peak 3 was the (*R*)-configuration, which, although opposite in designation, is spatially the same as (*S*)-α-hydroxy-terminal glycines normally generated by

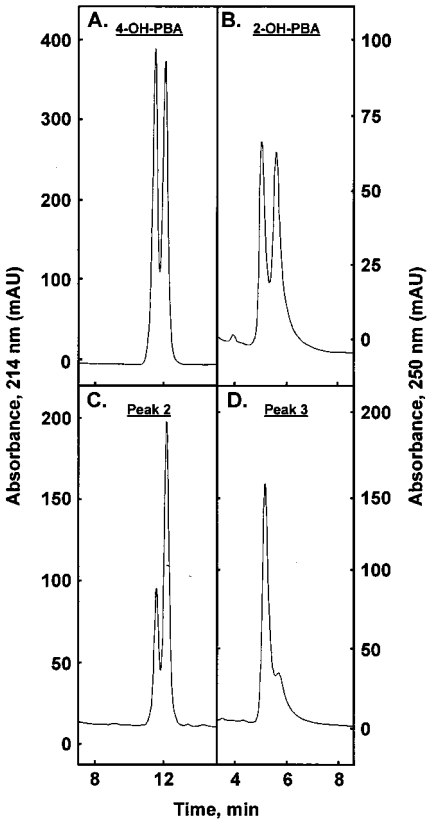

FIGURE 9: Separation of 4-OH-PBA and 2-OH-PBA stereoisomers by chiral chromatography. Separation of 4-OH-PBA and 2-OH-PBA enantomers was performed on Chirose C1 and Chirex COO5 columns, respectively, under isocratic conditions as described under Materials and Methods. Panel A, racemic mixture of synthetic 4-OH-PBA; panel B, racemic mixture of synthetic 2-OH-PBA; panel C, enzymatically generated peak 2 product isolated by reverse-phase HPLC; panel D, enzymatically generated peak 3 product isolated by reverse-phase HPLC.

PHM. Chiral assignment of the peak 2 product enantiomers was not possible due to the lack of an analogous stereospecific standard. It is important to note that following incubation of (*R*)-2-OH-PBA with either active or heat-denatured PHMcc, no (*S*)-enantiomer was detected, indicating that racemization does not spontaneously occur under the reaction conditions and that PHM itself does not catalyze the conversion of (*R*)-2-OH-PBA to (*S*)-2-OH-PBA. Taken collectively, these data indicate that both 4-OH-PBA and 2-OH-PBA are produced enzymatically and that the lack of absolute stereospecificity is likely due to limited rotational freedom afforded the small substrate within the binding pocket.

DISCUSSION

PBA is recognized as a potent, mechanism-based, irreversible inhibitor of PHM (*19*, *20*) and has been used as a tool for investigating both the mechanism and regulation of this essential enzyme. As a mechanism-based inhibitor, PBA is thought to mediate PHM inactivation by covalent modification of the protein (*20*, *29*, *37*). Accordingly, we sought to use PBA for detecting disulfiram-induced modification of the active site. However, when reacted with highly purified recombinant PHMcc in the presence of required cofactors, [^{3}H]PBA failed to generate specifically labeled protein. The assumption that PBA would label PHM was based upon previous observations that radioactivity from [^{14}C]PBA, similarly reacted with pituitary PHM, comigrated with the enzyme through gel exclusion and ion exchange chromatography (*29*). In our experiments, we also observe that a small amount of radioactivity co-chromatographs with recombinant PHM protein through HPLC; however, we found that this apparent labeling of PHM by PBA was completely independent of catalytic activity. Related observations have been made with PBA derivatives (*38*) and a vinyl glycine peptide (*39*) which irreversibly inactivated PHM without specifically labeling the protein. We therefore conclude that the observed association of PBA with enzyme represents a nonspecific interaction.

Our results demonstrate that the inactivation of PHM by PBA is functionally linked to the hydroxylation of PBA. Surprisingly, two hydroxylated products result from the action of PHM on PBA: 2-hydroxy-4-phenyl-3-butenoic acid (2-OH-PBA) and 4-hydroxy-4-phenyl-2-butenoic acid (4-OH-PBA). Under saturating conditions, a single molecule of PHM will hydroxylate approximately 100 molecules of PBA before inactivation inevitably occurs. The ability of PHM to hydroxylate PBA on both the α- and γ-carbons may be explained by a mechanism involving free radical resonance (Figure 10). We propose that the superoxide formed at CuB abstracts hydrogen from the substrate α-carbon to generate copper-bound hydroperoxide and a substrate radical. Resonance distributes the radical reactivity to the γ-carbon. Depending upon the positioning of the substrate relative to the hydroperoxide, the α- or γ-carbon could be in closer proximity to the activated oxygen bound at CuB. The resonating substrate radical can then attack the copper-bound hydroperoxide at either the oxygen atom immediately adjacent to CuB or the terminal oxygen atom. In the former case, hydroxylation proceeds through the formation of a copper−alkoxide intermediate, as has been proposed for the hydroxylation of dopamine by dopamine β-monooxygenase (*40*, *41*). In the latter case, hydroxylation occurs directly without an intermediate. In both instances, protonation from solvent regenerates free oxidized CuB. While Figure 10 shows the generation of 4-OH-PBA via the alkoxide pathway, it is equally possible that 2-OH-PBA could be generated via this intermediate and 4-OH-PBA via direct hydroxylation. Alternatively, both products could arise through a single mechanism.

Hydroxylation of glycine-extended peptides by PHM exhibits absolute stereospecificity with only the (*S*)-conformation being generated (*36*). X-ray crystallographic analysis has provided remarkable insight into the basis for this stereospecificity. Hydrogen bonding interaction between the oxygen of N316 and the amide hydrogen of the terminal glycine fixes the position of a natural substrate so that the activated oxygen at CuB can attack from only one angle (*9*). In contrast, PBA lacks this hydrogen bonding capability. The fact that PBA hydroxylation does not proceed in an absolutely stereospecific manner suggests that this substrate experiences considerable freedom of movement within the catalytic cleft. The predominant form of 2-OH-PBA produced by PHMcc is the (*R*)-configuration (80%), which, despite its opposite designation, actually corresponds to the orientation of hydroxylated glycine-extended substrates. The enantiomer, (*S*)-2-OH-PBA, constitutes 20% of this PBA product. Stereospecificity of hydroxylation at the 4-position is even

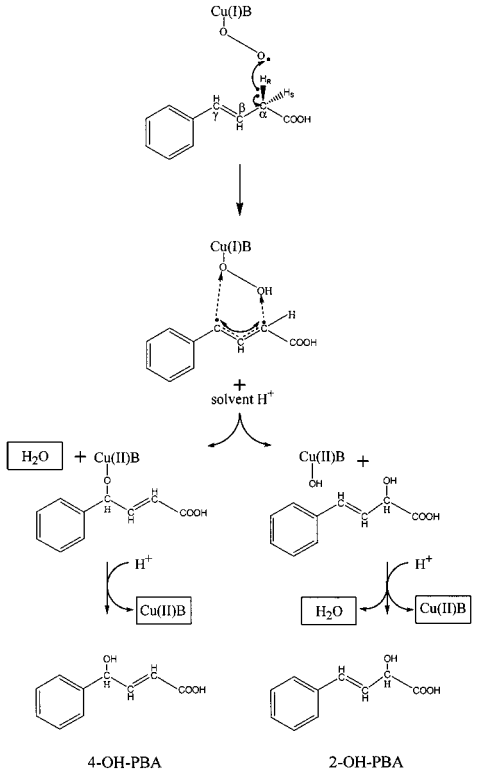

FIGURE 10: Alternative mechanisms proposed for the hydroxylation of PBA by PHM. Superoxide, formed at CuB, abstracts hydrogen from the α-carbon of PBA to generate copper-bound hydroperoxide and a substrate radical. Resonance distributes the radical reactivity to the γ-carbon. The resonating substrate radical can then attack the copper-bound hydroperoxide at either the oxygen atom immediately adjacent to CuB or the terminal oxygen atom. In the former case, the reaction proceeds through a copper−alkoxide intermediate with the concurrent generation of water. In the latter case, hydroxylation of the substrate occurs directly with the formation of Cu(II)B−hydroxyl. For both pathways, protonation from solvent then regenerates free oxidized CuB. The generation of 2-OH-PBA vs 4-OH-PBA reflects the orientation of substrate and copper-bound hydroperoxide. The extent to which a single mechanism, or a combination of the two proposed mechanisms, contributes to the generation of PBA products remains to be determined. It should be noted that the *pro-R* hydrogen in PBA is equivalent to the *pro-S* hydrogen for terminal glycine substrates.

less rigorous (70%:30%), indicating a greater freedom of movement at this carbon atom. This mechanism is distinct from "mirror-image catalysis" proposed for the nonstereospecific abstraction of hydrogen from dopamine by plasma amine oxidase (*42*). Assignment of chiral designations for the 4-OH-PBA enantiomers was not possible due to the lack of a stereospecific standard. It is important to note that the ratios between respective enantiomers were constant from experiment to experiment and under conditions of varying oxygen tension.

While these observations support the conclusion that the hydroxylation of PBA proceeds enzymatically and utilizes molecular oxygen as for peptide substrates, it is possible that some hydroxylation occurs via the addition of water to the radical intermediate. This pathway could account for the lack of absolute stereospecificity observed and provide a mechanism for PHM inactivation. Release of the PBA radical from the catalytic site prior to hydroxylation would leave activated oxygen at CuB without substrate, thus setting the stage for autoxidation. Our ongoing investigations indicate that the structure of the protein is altered following inactivation by PBA, possibly through the addition of hydroxyl groups.

Because PHM is critical to the activation of a large number of peptide messengers, inhibitory compounds have been actively sought (*3*, *43*). PBA is unique among these substances in being both a non-glycyl substrate and an irreversible, mechanism-based inactivator. The only other non-glycyl substrate for PHM hydroxylation reported to date, glyoxylate phenylhydrazone, does not inactivate PHM (*44*). The ability of PBA and glyoxylic acid phenylhydrazone to serve as PHM substrates indicates that a terminal glycine residue is not an absolute requirement for hydroxylation by PHM. This raises the possibility that natural PHM substrates may exist whose hydroxylated products are distinct from those involved in the formation of α-amidated messengers.

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