Novel Substrates and Inhibitors of Peptidylglycine α-Amidating Monooxygenase

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ABSTRACT: Peptidylglycine α-amidating monooxygenase (PAM, EC 1.14.17.3) catalyzes the formation of α-amidated peptides from their glycine-extended precursors, thus playing a key role in the processing of peptide neurohormones. We now report that PAM readily catalyzes three alternate monooxygenase reactions—sulfoxidation, amine N-dealkylation, and O-dealkylation. Thus, (4-nitrobenzyl)thioacetic acid is converted to the analogous sulfoxide, *N*-(4-nitrobenzyl)glycine is converted to 4-nitrobenzylamine and glyoxylate, and [(4-nitrobenzyl)oxy]acetic acid is converted to 4-nitrobenzyl alcohol and glyoxylate. All these new activities display the characteristics expected for the normal PAM-catalyzed reductive oxygenation pathway and produce an equimolar amount of glyoxylate together with the heteroatom-containing dealkylation products. The ester [(4-methoxybenzoyl)oxy]acetic acid is not a PAM substrate, but is instead a good competitive inhibitor (K_I = 0.48 mM). In addition, we report that the olefinic substrate analogues *trans*-benzoylacrylic acid and 4-phenyl-3-butenoic acid are potent time-dependent inactivators of PAM, with inactivation exhibiting the characteristics expected for mechanism-based inhibition. Monoethyl fumarate is also a time-dependent inactivator of PAM. Finally, we introduce several small non-peptide substrates for PAM by demonstrating that PAM catalyzes the transformation of hippuric acid and several ring-substituted derivatives to the corresponding benzamides and glyoxylic acid, with the most facile substrate of this class being 4-nitrohippuric acid. These compounds are the smallest amide substrates yet reported for PAM, and it is thus apparent that only the minimal structure of an acylglycine is required for PAM-catalyzed oxygenative amidation.

Bioactive neuropeptides are synthesized by various post-translational modifications of precursor peptides, which are themselves derived from direct gene translation products. A striking feature, necessary for the bioactivity of ~50% of known peptide neurohormones, is the presence of an α-amide group on the carboxy terminal end of the polypeptide chain. Peptidylglycine α-amidating monooxygenase (PAM, EC 1.14.17.3) catalyzes the formation of a host of α-amidated peptides from their glycine-extended precursors. PAM has been identified in a variety of tissues (Bradbury & Smyth, 1987a; Eipper & Mains, 1988; Sakata et al., 1986) and is the presumed in vivo catalyst of neuropeptide α-amidation. The enzyme has been isolated from various sources (Murthy et al., 1986; Kizer et al., 1986; Mehta et al., 1988; Mizuno et al., 1986), and cloned and sequenced (Eipper et al., 1987; Oshuye et al., 1988).

PAM is a copper-dependent and ascorbate-requiring monooxygenase which catalyzes the oxidative cleavage of terminal glycine to produce a C-amidated peptide and glyoxylic acid (Bradbury et al., 1982; Eipper et al., 1983). The enzyme stereospecifically abstracts the *pro-S* hydrogen of glycine (Ramer et al., 1988), consistent with the previous observation that although PAM has a high specificity for terminal glycine and does not catalyze the amidation of peptides terminating in other L-amino acids, peptides terminating with D-alanine are also slow substrates (Landymore-Lim et al., 1983). Recently, PAM was shown to catalyze the amidation of an α-hydroxyglycine-terminating peptide, in a manner independent of copper and ascorbate (Young & Tamburini, 1989). This was interpreted as evidence that α-hydroxyglycine is an intermediate along the pathway of PAM catalysis at a point subsequent to the oxygen-transfer step. Such a carbinolamine is a likely intermediate common to a number of possible mechanistic schemes for PAM catalysis.

Although the detailed mechanism of action of PAM is yet to be established, this enzyme is strikingly similar to dopamine β-monooxygenase (DBM, EC 1.14.17.1), another copper- and ascorbate-requiring monoxygenase. While the biological function of DBM is apparently benzylic hydroxylation of dopamine to produce norepinephrine, work in our laboratory has demonstrated several new kinetically facile monooxygenase activities for DBM—stereoselective sulfoxidation of phenyl aminoalkyl sulfides (May & Phillips, 1980; May et al., 1981a) and selenoxidation of selenides (May et al., 1987), oxygenative ketonization of benzylic S-alcohols (May et al., 1981b, 1982), epoxidation of olefins (May et al., 1983; Padgette et al., 1985), alkyne oxidation (Padgette et al., 1985), and allylic hydroxylation (Sirimanne & May, 1988) and aromatization (Wimalasena & May, 1989). Mechanistic studies and the development of DBM inhibitors have also been carried out in other laboratories (Steward & Klinman, 1988; Fitzpatrick & Villafranca, 1987). Our laboratory recently reported that DBM readily catalyzes oxygenative N-dealkylation, and we demonstrated that the mechanism involves initial electron abstraction to form the nitrogen cation radical (Padgette et al., 1985; Wimalasena & May, 1987). This N-dealkylation activity of DBM is chemically analogous to the amidation reaction catalyzed by PAM, thus underscoring the similarity between these two enzymes.

We now report that PAM indeed readily catalyzes three such alternate monooxygenase reactions—sulfoxidation, amine N-dealkylation, and O-dealkylation. In addition, we report that several olefinic substrate analogues, including 4-phenyl-3-butenoic acid, *trans*-benzoylacrylic acid, and monoethyl fumarate, are potent turnover-dependent inactivators of PAM, with inactivation exhibiting the characteristics expected for mechanism-based inhibition. Finally, we report that PAM readily catalyzes the oxidative cleavage of hippuric acid to benzamide and glyoxylic acid. This is the smallest amide substrate yet reported for PAM, and it is thus apparent

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that only the minimal structure of an acylglycine is required for active site turnover.

Experimental Procedures

Materials and Methods. Materials. Ascorbic acid and all buffers were obtained from Sigma Chemical Co. Beef liver catalase (65000 U/mg) was from Boehringer Mannheim. Trinitrobenzenesulfonic acid (TNBSA) and Coomassie Blue protein assay mix were from Pierce. All amino acids and peptides were from Bachem (Torrance, CA). All chemicals used for synthesis were from Aldrich. Hippuric acid, 4-nitrohippuric acid, 4-phenyl-3-butenoic acid (styrylacetic acid), and monoethyl fumarate were from Aldrich and were crystallized before use. HPLC analyses were performed on a Spherisorb C8 reversed-phase column (Altech), using a LDC Constametric III system outfitted with a LDC Spectromonitor 3100 variable-wavelength detector. GC analyses were performed using a 30-m DB5-30W 0.25-μM open tubular column (J&W Scientific) in a Hewlett-Packard 5890 gas chromatograph, mass spectra were obtained on a VG 70SE mass spectrometer, and UV/vis measurements were obtained with a Hewlett-Packard 8451A diode array spectrophotometer.

Enzyme Isolation and Assay. Enzyme isolations were performed at 4 °C using a modification of the procedure described by Murthy et al. (1986). In a typical isolation, 50 frozen bovine pituitaries (Pel Freeze Biologicals) were dissected and the neurointermediate fraction was homogenized (1 min with a hand-held homogenizer) in 200 mL of 25 mM Na-TES, pH 8.5. The solution was centrifuged (45000*g* for 30 min) and the supernatant was subjected to ammonium sulfate precipitation. The 25–45% ammonium sulfate pellet was suspended in 10 mL of 100 mM Na-TES, pH 7.0, and centrifuged (100000*g* for 60 min). The supernatant solution was then frozen at −70 °C for later use. The frozen ammonium sulfate cut was thawed, diluted with 5 volumes of 20 mM Tris-HCl, pH 7.2, 2.5 mM imidazole, 100 mM NaCl buffer, and centrifuged (100000*g* for 30 min). The supernatant was loaded (50 mL/h) on a column packed with 10 mL of chelating Sepharose (Pharmacia) charged with Cu by passing 50 mL of 10 mg/mL $CuSO_4$ in water and equilibrated with the same buffer. The column was washed with starting buffer until A_{280} of the effluent went to base line and eluted by using a gradient of starting buffer and 50 mM imidazole hydrochloride, pH 7.0, total volume of the gradient was 140 mL, and flow rate was 24 mL/h. Fractions with PAM activity were pooled and concentrated in an ultrafiltration cell using an Amicon YM10 membrane. The concentrated material (approximately 5 mL) was diluted with 20 mL of 100 mM MES pH 6.6 buffer and then immediately loaded on a substrate affinity column equilibrated with the same buffer. The substrate affinity column was made by linking 10 mg of D-Tyr-Trp-Gly to 0.5 g of activated CH-Sepharose (Pharmacia) according to the instructions provided by the manufacturer. Loading was performed at a flow rate of 30 mL/h and the column was washed with 100 mM MES, 500 mM NaCl, pH 6.6, until the A_{280} of the effluent went to base line. The column was then eluted with 1 M imidazole hydrochloride, pH 8.5; 40 mL of imidazole wash was enough to elute all PAM activity. The material was concentrated and dialyzed with 100 mM Tris-HCl, pH 7.2, by use of an Amicon microdialysis apparatus. The dialyzed protein was assayed (typical specific activity was approximately 2700 nmol mg^{-1} h^{-1} under the assay conditions described below, and typical yields were approximately 0.1 mg of total protein) and stored in 50% ethylene glycol and 1 mg/mL BSA at −20 °C. This preparation, which contains a mixture of PAM A and B (Murthy et al., 1986), was used for all assays unless otherwise indicated.

PAM activity was assayed using the substrate *N*-(trinitrophenyl)-D-Tyr-Val-Gly (TNP-D-Tyr-Val-Gly), as reported previously (Katopodis & May, 1988). TNP-D-Tyr-Val-Gly was synthesized by a modification of our previously described procedure. Typically, 10 mg of D-Tyr-Val-Gly in 20 mL of 80/20 water/methanol was mixed with a 50% excess of TNBSA and triethylamine. The reaction mixture was stirred at room temperature for 30 min, 100 mL of water was added, the pH was adjusted to 4 with dilute HCl, and the mixture was extracted twice with ethyl acetate. The organic layer was dried with anhydrous sodium sulfate and evaporated in vacuo. The intensely yellow material was dissolved in a few drops of ethyl acetate and precipitated overnight with ethyl ether and hexane. The supernatant was decanted and the fine precipitate was dissolved in methanol and stored at −70 °C, where it is stable for at least 1 year. Assays were performed in a total volume of 0.500 mL of 100 mM Na-MES, pH 6.6, containing 4 mM ascorbate, 2 μM $CuSO_4$, 6500 U of catalase, and various amounts of enzyme. Incubations were at 37 °C in a shaking water bath and were initiated by the addition of 20 μL of methanolic solution of the appropriate substrate. Aliquots of 100 μL were obtained at various time intervals, quenched with 10 μL of 2 M H_2ClO_4, centrifuged to remove the protein, and analyzed by HPLC. In this manner, several time points were obtained for each assay, allowing us to confirm that the rates obtained were in the linear portion of turnover. TNP-D-Tyr-Val-NH_2 was detected by HPLC using a solvent system of 50% acetonitrile/50% 50 mM ammonium acetate, pH 5.5. The same system with lower ratios of organic phase was used for the detection of the various hippuric acid derivatives and their products. Detection was at 344 nm for TNP-peptides, 278 nm for 4-nitrohippuric acid and 4-nitrobenzamide, 259 nm for 4-methoxyhippuric acid and 4-methoxybenzamide, 243 nm for hippuric acid and benzamide, and 270 nm for all 4-nitrobenzyl substrates and products. Quantitation of products was performed by comparison of peak heights to standard curves constructed from serial dilutions of authentic materials using exactly the same HPLC elution conditions. All values reported were in the linear response range of the system. Values for V_{max} and K_m were obtained from the inverse plots by using a least-squares fit program. Typically data are accurate within 5% of the reported values.

Time-Dependent Inactivation of PAM. Inactivation of PAM by olefin inactivators was determined by the dilution method. Inactivation reactions containing 1 μM $CuSO_4$, 2 mM ascorbate, 2000 U of catalase, purified PAM, and various concentrations of inhibitor in a total volume of 0.200 mL of 100 mM Na-MES pH 6.6 buffer were incubated at 37 °C, and the reaction was initiated by the addition of enzyme. Aliquots of 20 μL were withdrawn, diluted into 1 mL of assay solution (2 μM $CuSO_4$, 4 mM ascorbate, 10000 U of catalase, 120 μM TNP-D-Tyr-Val-Gly), and incubated at 37 °C for 90 min. Aliquots of the assay solution were quenched with H_2ClO_4 and analyzed by HPLC as indicated above. The first-order inactivation constant was determined by linear regression analysis of the slopes of lines (k_{obsd}) resulting from the plot of ln (percent remaining activity) vs time. The plot of $1/k_{obsd}$ vs $1/[I]$ was linear, and k_{inact} was the calculated $1/(y$ intercept) and K_I the $-1/(x$ intercept).

For the dialysis experiment, two inhibition mixtures containing 5 mM inhibitor were prepared, one with 4 mM ascorbate and the other with no ascorbate. After incubation at 37 °C for 30 min, a 20-μL aliquot of each mixture was assayed. The rest of the inhibition mixtures were dialyzed at

4 °C against 300 mL of 100 mM Tris-HCl, pH 7.2, containing 10% ethylene glycol (two changes of dialysis buffer in 8 h). Aliquots of each dialyzed enzyme mix were reassayed as before.

Glyoxylic Acid Determination. Glyoxylic acid was determined by the spectrophotometric method of Christman et al. (1944), with minor modifications in order to adapt it to the requirements of the PAM assay. Assays were performed as indicated above; at the appropriate time the assay mix was quenched with 50 μL of 2 M H_2ClO_4 and centrifuged to remove protein. A 400-μL aliquot was mixed with 50 μL of phenylhydrazine hydrochloride (10 mg/mL of H_2O) and incubated at 37 °C for 5 min. The sample was then placed on ice and 200 μL of ice-cold 6 N HCl was added. After a 1-min incubation at 0 °C, 50 μL of ferricyanide (50 mg of ferricyanide/mL of H_2O) was added, the samples were incubated at 25 °C for 15 min, and absorbance at 520 nm was measured. Under these conditions color formation reaches its maximum level ~15 min after addition of ferricyanide, plateaus for approximately 10 min, and subsequently decreases slowly. It is therefore important that all absorbance measurements be performed within the 10-min plateau in absorbance change. Control assays with no substrate present were performed as a reference, and standard curves with known amounts of glyoxylate were used for product quantification.

Syntheses. 4-Methoxybenzoylglycine (4-Methoxyhippuric Acid) (**2**). A 10-g sample of 4-methoxybenzoyl chloride was dissolved in 100 mL of dry ethyl ether and mixed with 6.5 g of glycine ethyl ester (free amine form). Triethylamine (5 g) was added dropwise and the solution was stirred at room temperature for 1 h. Ethyl acetate (200 mL) was added and the organic layer was extracted twice with 100 mL of 0.5 M HCl, dried, and concentrated. The resulting oil was dissolved in 50 mL of methanol, and 75 mL of 1 M NaOH was then added. The solution was stirred at room temperature for 3 h, acidified, saturated with NaCl, and extracted with 300 mL of ethyl acetate. The organic layer was washed with water, dried, and concentrated. The solid was dissolved in warm ethyl acetate and crystallized overnight at −20 °C from a mixture of ethyl acetate/ether/hexane. The final yield was 7.8 g of white crystals: mp 176–178 °C; ^{1}H NMR (acetone-d_6) δ 7.91 (d, 2 H), 7.01 (d, 2 H), 4.12 (d, 2 H), 3.86 (s, 3 H); mass spectrum (EI) *m/e* 209 (M^+).

[(4-Methoxybenzoyl)oxy]acetic Acid (**4**). The cesium carbonate method of esterification was used (Wang et al., 1976). 4-Methoxybenzoic acid (5 g) was dissolved in 50 mL of water/methanol and the pH was adjusted to 7 with a 20% aqueous solution of Cs_2CO_3. The mixture was evaporated to dryness under vacuum and the residue reevaporated twice from 50 mL of DMF. The cesium salt obtained was stirred with 7.6 g of benzyl 2-bromoacetate in 50 mL of DMF for 6 h at room temperature. The solvent was evaporated in vacuo (water-bath temperature 40 °C), 100 mL of water was added and the product was extracted in two 100-mL volumes of ethyl acetate. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The solid was dissolved in 100 mL of methanol, and the benzyl protective group was removed by catalytic hydrogenation at atmospheric pressure using 0.5 g of Pd/C as a catalyst. After filtration of the catalyst and evaporation of the solvent the product was crystallized from ethyl ether/hexane; 3.7 g of crystals was obtained: mp 108 °C; ^{1}H NMR ($CDCl_3$) δ 8.03 (d, 2 H), 7.08 (d, 2 H), 4.88 (s, 2 H), 3.91 (s, 3 H); mass spectrum (CI) *m/e* 221 (M + 1).

(4-Nitrobenzyl)thioacetic Acid (**5**). A 10.0-g (46-mmol) sample of 4-nitrobenzyl bromide was dissolved in 200 mL of dry THF and 4.3 g (46 mmol) of mercaptoacetic acid was added. The solution was cooled in an ice bath and excess triethylamine was slowly dropped in with vigorous stirring. The solution was allowed to warm to room temperature and was stirred for 1 h. The solvent was evaporated and the solid was dissolved in 100 mL of 0.5 N NaOH. The aqueous phase was extracted twice with ethyl ether and was then acidified to pH 2.0 by the addition of HCl. The acidic solution was extracted twice with ethyl ether and the combined ethereal extracts were dried with Na_2SO_4 and concentrated to dryness; 9.5 g of a pale yellow solid was collected: mp 108 °C; ^{1}H NMR ($CDCl_3$) δ 8.21 (d, 2 H), 7.54 (d, 2 H), 3.94 (s, 2 H), 3.11 (s, 2 H); mass spectrum (CI) *m/e* 228 (M + 1).

(4-Nitrobenzyl)thionylacetic Acid. A 2.0-g (9-mmol) sample of **5** was dissolved in 10 mL of methanol and cooled to 4 °C. An ice-cold solution of 2.1 g (10 mmol) of sodium periodate in 10 mL of water was added and the mixture was allowed to stand overnight in the cold. The white precipitate was filtered and 100 mL of ethyl acetate was added. The organic phase was washed with 0.1 N HCl, 10% Na_2SO_3, and water, dried over Na_2SO_4, and evaporated to dryness. About 1.5 g of the pale yellow sulfoxide solid was collected: mp 123–124 °C; ^{1}H NMR (acetone-d_6) δ 8.30 (d, 2 H), 7.72 (d, 2 H), 4.52 (d, 1 H), 4.32 (d, 1 H), 3.92 (d, 1 H), 3.64 (d, 1 H); mass spectrum (CI) *m/e* 244 (M + 1).

N-(4-Nitrobenzyl)glycine (**6**). A 9.6-g (93-mmol) sample of glycine ethyl ester (free base form) was dissolved in 200 mL of dry THF and cooled in an ice bath, and 10 g of 4-nitrobenzyl bromide (46.3 mmol, dissolved in 100 mL of dry THF) was slowly added. The mixture was stirred overnight at room temperature, and the THF was evaporated under reduced pressure. The solid was dissolved in 50 mL of methanol, 200 mL of cold 1 M NaOH was added, and the mixture was stirred for 2 h at room temperature. The solution was extracted twice with ethyl ether, and the pH of the clear yellow aqueous phase was adjusted to 7.0 by the dropwise addition of concentrated HCl with vigorous stirring. The milky solution was cooled overnight, and the pale yellow solid was filtered and washed with water. After drying under vacuum, 8.5 g of solid was collected: mp 198 °C (turned dark brown upon melting); ^{1}H NMR (D_2O) δ 10.7 (m, 2 H), 10.1 (m, 2 H), 6.8 (s, 2 H), 6.1 (s, 2 H); mass spectrum (EI) *m/e* 210 (M^+).

[(4-Nitrobenzyl)oxy]acetic Acid (**7**). A mixture of 10 g of 4-nitrobenzyl alcohol (65.2 mmol), 15 g of methyl bromoacetate (98 mmol), 50 g of anhydrous K_2CO_3, and 1 g of *N*-(*t*-Bu)$_4$Br was refluxed under N_2 with vigorous stirring in 300 mL of dry THF. The reaction progress was monitored by HPLC. After 3 days of reflux the reaction mixture darkened considerably, and ~80% of the 4-nitrobenzyl alcohol had reacted. The solid was removed by filtration, the THF was evaporated under vacuum, the remaining material was dissolved in ethyl ether, and the ether layer was washed two times with water. The ether was evaporated and 200 mL of cold 1 M NaOH was added to the yellow oil. After being stirred at room temperature for 2 h, the solution was extracted five times with ethyl ether. HPLC monitoring of the ether layer indicated that the last ether washes contained no 4-nitrobenzyl alcohol. The aqueous layer was cooled and acidified to pH 2 with concentrated HCl. The product oiled out, and several attempts to crystallize it failed. The aqueous layer was then extracted with ethyl acetate twice, and the organic phase was dried over Na_2SO_4 and evaporated under

Table I: Kinetic Parameters of PAM Substrates[a]

substrate	product[b]	K_m (mM)	K_I (mM)	V_{max} (nmol mg^{-1} h^{-1})
4-NO_2-C_6H_4-C(=O)$NHCH_2CO_2H$ (**1**)	4-NO_2-C_6H_4-C(=O)NH_2	1.0		1370
4-CH_3O-C_6H_4-C(=O)$NHCH_2CO_2H$ (**2**)	4-CH_3O-C_6H_4-C(=O)NH_2	1.6		493
C_6H_5-C(=O)$NHCH_2CO_2H$ (**3**)	C_6H_5-C(=O)NH_2	1.7		109
4-CH_3O-C_6H_4-C(=O)OCH_2CO_2H (**4**)	none		0.48	
4-NO_2-C_6H_4-$CH_2SCH_2CO_2H$ (**5**)	4-NO_2-C_6H_4-CH_2S(=O)CH_2CO_2H	0.34		2310[c]
4-NO_2-C_6H_4-$CH_2NHCH_2CO_2H$ (**6**)	4-NO_2-C_6H_4-CH_2NH_2	1.8		2930
4-NO_2-C_6H_4-$CH_2OCH_2CO_2H$ (**7**)	4-NO_2-C_6H_4-CH_2OH	0.16		1360
C_6H_5-C(=O)CH=$CHCO_2H$ (**8**)			0.16	3.6 min^{-1} (k_{inact})
TNP-D-Tyr-Val-Gly	TNP-D-Tyr-Val-NH	0.013		2580

[a] Kinetic values were obtained by using the standard PAM assay with detection of the appropriate products by HPLC; see Experimental Procedures for conditions. [b] This column presents only the HPLC detectable products. Glyoxylate was also detected spectrophotometrically in all cases of dealkylation. [c] This value is for sulfoxide formation and does not take into account any S-dealkylation product.

vacuum; 6.7 g of a pale yellow solid was collected: mp 114–115 °C; ^{1}H NMR (acetone-d_6) δ 8.27 (m, 2 H), 7.7 (m, 2 H), 4.81 (s, 2 H), 4.28 (s, 2 H); mass spectrum (CI) *m/e* 212 (M + 1).

[(*4-Methoxybenzyl*)*oxy*]*acetic Acid.* A 1.0-g sample of Na (43.5 mmol) was dissolved in 50 mL of methanol and 6.0 g (43.5 mmol) of 4-methoxybenzyl alcohol was added. The methanol was evaporated and the solid was kept under high vacuum for 1 h. Dry THF (100 mL) was added, and 8.0 g of methyl bromoacetate (52.3 mmol) dissolved in 100 mL of dry THF was added slowly and with vigorous stirring. The reaction mixture was stirred for 5 h, the solvent was removed under vacuum, and 200 mL of cold 1 M NaOH was added to the colorless oil. After being stirred at room temperature for 2 h, the aqueous layer was extracted three times with ethyl ether, then acidified with cold concentrated HCl to pH 2, and extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated; 5.8 g of a colorless oil was collected: ^{1}H NMR ($CDCl_3$) δ 7.3 (m, 2 H), 6.9 (m, 2 H), 4.6 (s, 2 H), 4.12 (s, 2 H), 3.82 (s, 3 H); mass spectrum (CI) *m/e* 196 (M + 1).

trans-Benzoylacrylic Acid (**8**). Synthesis was performed as reported by Grummitt et al. (1962). Starting with 17 g of maleic anhydride, 19 g of crystalline product was obtained after the first crystallization: mp 95–96 °C; ^{1}H NMR (acetone-d_6) δ 8.08 (m, 2 H), 7.95 (d, 1 H), 7.71 (m, 1 H), 7.60 (m, 2 H), 6.80 (d, 1 H); mass spectrum (EI) *m/e* 176 (M^+).

3-Benzoyl-2,3-epoxypropionic Acid. This epoxide was synthesized by a modification of the method of Wasson and House (1963). A 2.2-g (12.5-mmol) sample of **8** was dissolved in 50 mL of methanol and 5 mL of 30% H_2O_2 was added. The solution was cooled to 15 °C and 19 mL of 1 M NaOH was slowly added. The solution temperature was kept between 15 and 20 °C. After addition of base was completed, the solution was slowly brought to 25 °C and allowed to stir for 3 h. H_2O (100 mL) was added and the solution was acidified by addition of 1 M HCl to pH 2.5. It was then extracted with 200 mL of ethyl acetate and the organic phase, after being dried over Na_2SO_4, was evaporated to dryness; 1.7 g of a white solid was collected: mp 95 °C; ^{1}H NMR (acetone-d_6) δ 8.14 (m, 2 H), 7.74 (m, 1 H), 7.63 (m, 2 H), 4.65 (d, 1 H), 3.67 (d, 1 H); mass spectrum (CI) *m/e* 193 (M + 1). The diol 3-benzoyl-2,3-dihydroxypropanoic acid was prepared by incubating 100 mg of 3-benzoyl-2,3-epoxypropanoic acid in 5 mL of 0.1 M sulfuric acid for 30 min. The solution was then neutralized and the diol was used as an HPLC standard.

Results

Hippuric Acid Substrates. We examined a series of hippuric acids as potential PAM substrates, since these compounds, being N-blocked glycines, represent minimal amidation substrates for the enzyme. 4-Nitrohippuric acid (**1**) was examined first because the expected product has a high extinction coefficient and would therefore be the easiest to detect by HPLC. Incubations of **1** with either bovine pituitary extracts or purified PAM produced 4-nitrobenzamide in an enzyme-dependent manner. Product formation is strictly ascorbate and copper dependent as expected for PAM-catalyzed monooxygenation. Product formation is linear with respect to enzyme concentration, increases linearly with time (followed up to 4 h), and is abolished when the enzyme sample is boiled for 10 min before assay. Enzymatically produced 4-nitrobenzamide coeluted with an authentic standard when analyzed by two different solvent systems on HPLC. To further establish that 4-nitrobenzamide is indeed produced via a PAM-catalyzed oxygenative process, we examined the stoichiometry of formation of this product vs glyoxylate, the other obligatory product of amidation by PAM. Using the spectrophotometric assay described under Experimental Procedures, we determined a ratio of 0.93/1 for glyoxylate vs 4-nitrobenzamide, demonstrating that indeed approximately one molecule of glyoxylate is produced for every molecule of 4-nitrobenzamide.

Similar experiments with 4-methoxyhippuric acid and hippuric acid established that these molecules are also PAM substrates and require the complete PAM system for turnover. Both glyoxylate and the corresponding amide product were detected from the reaction of each of these compounds with PAM. For both substrates identification of the benzamide products was accomplished on the basis of HPLC coelution with an authentic standard. In the case of hippuric acid, in addition to HPLC detection of the benzamide product, we were able to obtain GC/MS confirmation of its identity. Enzymatic assays using hippuric acid as a PAM substrate were quenched with perchlorate, the protein was removed by centrifugation, and the pH was adjusted to 8.0. The samples were saturated with NaCl and extracted with ethyl acetate, and the organic layer was analyzed by GC. Only the complete PAM system produced a peak that coeluted with authentic benzamide and had a mass spectrum identical with that of the authentic material (M^+ = *m/e* 121). The substrate kinetic data for all three hippuric acid substrates produced linear inverse plots and the kinetic parameters calculated are displayed in Table I.

Inhibition by [(*4-Methoxybenzoyl*)*oxy*]*acetic Acid.* In order to probe the role of the amide nitrogen in PAM catalysis, we examined the ester analogues of the hippuric acid substrates. We first examined [(4-nitrobenzoyl)oxy]acetic acid, since the analogous amide is the best hippuric acid substrate. Under our assay conditions however, [(4-nitrobenzoyl)oxy]-acetic acid exhibited a high rate of nonenzymatic hydrolysis, which precluded meaningful enzymatic assays. We therefore turned to [(4-methoxybenzoyl)oxy]acetic acid (**4**), which is resistant to nonenzymatic hydrolysis under normal assay conditions. Several incubations of **4** with the complete purified

PAM system produced no detectable 4-methoxybenzoic acid product even after 2 h of incubation. Parallel experiments with the amide analogue, 4-methoxyhippuric acid, under the same conditions produced high amounts of 4-methoxybenzamide. These results establish that PAM is not capable of oxidative cleavage of the ester substrate analogue **4**. Competition kinetics of **4** vs TNP-D-Tyr-Val-Gly were performed, to determine whether the ester is an inhibitor of PAM. The linear double-reciprocal plots obtained and the linear slope vs [I] replot indicated straightforward competitive inhibition. Analysis of the slope of the secondary plot yielded a K_I of 0.48 mM.

It should be noted that when ester **4** was incubated with crude pituitary homogenates, a small amount of 4-methoxybenzoic acid was produced, corresponding to approximately $^1/_3$ of the activity of the analogous amide. Production of 4-methoxybenzoic acid by the crude homogenate was independent of either copper or ascorbate, indicating that product formation is not the result of a PAM-type monooxygenase activity. As an additional test for oxygenase activity, parallel assays were performed with both the purified and the crude systems, using spectrophotometric detection of glyoxylate. No detectable glyoxylate was formed in either case. We therefore concluded that the small amount of ester cleavage seen in the crude system arises from an esterase-type activity present in crude pituitary homogenates.

Novel Monooxygenase Activities of PAM. In order to ascertain whether the catalytic competence of PAM extends to reactions other than amidation, we examined several small molecules designed to reveal additional reactivities for this enzyme. Incubations of the prototypical sulfur substrate (4-nitrobenzyl)thioacetic acid (**5**) with the complete PAM system produced (4-nitrobenzyl)thionylacetic acid in an enzyme-, ascorbate-, and copper-dependent manner. Enzymatically produced sulfoxide was first identified by coelution with authentic sulfoxide on HPLC. Subsequently, large-scale enzymatic production of sulfoxide was performed in order to obtain enough material for direct structural confirmation by NMR. The assay amounts were increased by 100-fold, and after overnight incubation at 37 °C, the solution was acidified to pH 2.0 and protein was removed by centrifugation. The solution was then extracted three times with 10 mL of hexane, saturated with NaCl, and extracted three times with 10 mL of chloroform. The combined chloroform extracts were evaporated to dryness and the residue was washed with a small amount of hexane. HPLC of the resulting residue, using UV detection at 278 nm, established formation of a product that coeluted with authentic sulfoxide. The NMR spectrum of the enzymatic product contained the characteristic AB AB pattern and phenyl region of authentic sulfoxide, thus confirming that the enzymatic product is indeed (4-nitrobenzyl)thionylacetic acid. In kinetic experiments, incubations of purified PAM with **5**, using HPLC detection of sulfoxide, gave rise to normal saturation kinetics and the parameters calculated from the inverse plots are listed in Table I. Several attempts to determine the enantiomeric purity of enzymatically produced sulfoxide failed. A chiral Pirkle HPLC column failed to separate the optical isomers of authentic material and various europium shift reagents were also unsuccessful in separating the NMR signals of the protons α to the sulfoxide.

We examined the possibility that PAM may also catalyze oxygenative S-dealkylation of **5**, in addition to sulfoxidation. Assays using HPLC detection failed to detect any 4-nitrobenzyl mercaptan, the expected heteroatom containing product from S-dealkylation of **5**. However, when we examined assay solutions of **5** for glyoxylate production, a small but significant amount of glyoxylate was consistently detected. Parallel assays in which both glyoxylate and sulfoxide were quantitated indicated a ratio of approximately 8/1 for sulfoxide to glyoxylate produced. This result confirms that a small amount of oxidative S-dealkylation does occur with substrate **5**.

Novel substrate analogues of **5** containing heteroatoms other than sulfur were also examined as possible PAM substrates. Assays of *N*-(4-nitrobenzyl)glycine (**6**) with purified PAM produced both 4-nitrobenzylamine and glyoxylic acid. Product formation is strictly dependent on the presence of ascorbate and active enzyme. The pH maximum for (4-nitrobenzyl)-glycine turnover is 8.5 and activity with this substrate rapidly declines on either side of this pH value. In our hands, all uncharged PAM substrates have a pH maximum around 6.6. The high pH maximum of **6** likely reflects a balance between declining enzymatic activity but increasing availability of uncharged amine with increasing pH. Interestingly, despite the high pH maximum of this substrate activity, the V_{max} value obtained is higher than any other substrate reported here.

Similar assays were performed with the ether analogue [(4-nitrobenzyl)oxy]acetic acid (**7**). This compound is a good PAM substrate, producing 4-nitrobenzyl alcohol and glyoxylic acid only when the complete functional PAM system is present. The ratio of glyoxylic acid to alcohol product was determined in parallel assays and was 1.2/1. On the basis of substrate kinetic parameters obtained by following production of 4-nitrobenzyl alcohol product, **7** is the best small molecule substrate reported here (Table I), despite the fact that it lacks the carbonyl moiety present in the amide substrates. In further experiments, we also examined [(4-methoxybenzyl)oxy]acetic acid, which is the ether analogue of **4**. This ether is also a good PAM substrate and produced both 4-methoxybenzyl alcohol and glyoxylate only when the complete functioning PAM system was present.

Inactivation by Olefinic Compounds. We anticipated the possibility that olefinic substrate analogues might represent mechanism-based irreversible inhibitors for PAM, as is the case for DBM (May et al., 1983; Padgette et al., 1985) and P_{450} enzymes (Ortiz de Montellano et al., 1982). Accordingly we examined *trans*-benzoylacrylic acid (**8**) as a potential turnover-dependent inactivator of PAM. In order to determine the extend to which PAM catalyzes epoxidation of **8**, this compound was incubated with highly active purified or crude PAM and the reaction mixtures were examined by HPLC for formation of the epoxide product, 3-benzoyl-2,3-epoxypropionic acid, or the corresponding diol, 3-benzoyl-2,3-dihydropropanoic acid. Authentic standards of both epoxide and diol were synthesized as described under Experimental Procedures. Numerous assays of **8** with PAM produced no detectable formation of either epoxide or diol product. It was immediately evident, however, that **8** is a potent, time-dependent inactivator of PAM. Kinetic parameters for the inactivation reaction were determined by the dilution assay method; inactivation is strictly first order and concentration dependent, and the double-reciprocal plot of 1/rate vs 1/[I] gives the values of k_{inact} = 3.6 min^{-1} and K_I = 0.16 mM (Table I). Inactivation by **8** is strictly dependent on the presence of ascorbate in the inactivation solution, and addition of the substrate, 4-nitrohippuric acid, in the inactivation solution protects against inactivation. PAM inactivated (6% of initial activity) with **8** did not recover enzymatic activity even after extensive dialysis. Under the same incubation and dialysis conditions a control solution, with no ascorbate added, retained 79% of predialysis activity. Although no epoxide product was detected, we performed

additional experiments to determine if a small amount of enzymatically produced epoxide is responsible for the observed inactivation of the enzyme. PAM incubated with 0.285 mM 3-benzoyl-2,3-epoxypropionic acid (either with or without ascorbate) retained its full activity even after 30 min of incubation.

Several other olefins were examined for inhibition of PAM. We find that 4-phenyl-3-butenoic acid is a potent time-dependent inactivator of PAM. Inhibition is strictly ascorbate dependent and hippuric acid substrates protect from inhibition. The apparent K_I for this inhibitor is near 1 μM, which is $\sim$3 orders of magnitude lower than for olefin **8**. Thus, concentration dependence of inhibition by 4-phenyl-3-butenoic acid can only be observed at very low inhibitor concentrations. Also, deviations from the standard first-order behavior of mechanism-based inhibition occur. We calculate a k_{inact}/K_I value for this inhibitor of approximately 6700 mM^{-1} min^{-1}, which indicates that this compound is an extremely potent PAM inactivator. Monoethyl fumarate is also a PAM inactivator (k_{inact} = 3.2 min^{-1}, K_I = 1.3 mM), demonstrating that the phenyl ring in **8** is not necessary for inactivation of the enzyme. Finally, CBZ-dehydroalanine, even after extended incubations, does not inhibit PAM.

Discussion

We report here a series of hippuric acids as the smallest amidation substrates yet found for PAM. These compounds are N-blocked glycines and in this sense represent a minimal amidation substrate for the enzyme. The ability of PAM to process such small substrates indicates that the catalytic site can bind and turn over substrates independently of any extended peptide recognition site. While the small molecules presented here have less favorable K_m values than peptide substrates, they offer a great advantage in flexibility of design and ease of synthesis. In addition, small molecule substrate and inhibitors may be less susceptible to in vivo degradation and may cross granule membranes better than peptides, thus making them useful for cellular level experiments. It should be noted that Bradbury and Smyth (1987b) have reported that hydrazones of glyoxylic acid are substrates for PAM, being converted to the corresponding oxalic acid hydrazides, and Kizer et al. (1986) have reported on the inhibition of PAM by substrate analogues such as acetopyruvate.

As shown in Table I, substituted hippuric acids have K_m values similar to each other, which are, however, approximately 2 orders of magnitude higher than the K_m for the tripeptide substrate. The best hippuric acid substrate is the 4-nitro-substituted derivative, which has a V_{max} value $^1/_2$ that of TNP-D-Tyr-Val-Gly. Interestingly there is a sizable difference in the substrate reactivity of hippuric acids, depending on ring substitution. This is evident in the V_{max} values shown in Table I and is more pronounced if V_{max}/K_m is considered. The three hippuric acid substituents shown here span the range from strongly electron withdrawing to strongly electron donating and have, therefore, profound differences in both the electron density at the amide nitrogen and the possible stabilization or destabilization of charge buildup at the benzoyl position during the catalytic cycle (March, 1985). It would therefore be expected that electron transfer from the hippuric acid amide bond would be strongly affected by para substitution. We emphasize that it is not yet clear to what extent the V and V/K values reported here reflect the intrinsic rate of the oxygenation process; obviously, detailed kinetic analysis of para-substituted hippuric acids will help define the role of the amide bond during oxidation by PAM.

In order to better understand the role of the amide moiety during PAM catalysis, we examined the reactivity of several analogues of hippuric acid with PAM. In the cases of DBM- and P_{450}-catalyzed reactions, there is strong evidence that sulfoxidation and N-dealkylation proceed through the intermediacy of a species with cationic and/or radical character, generated by initial abstraction of a single electron from the heteroatom (Wimalasena & May, 1987; Watanabe et al., 1980, 1981; Augusto et al., 1982; Stearns & Ortiz de Montellano, 1985; Shea et al., 1982). On the other hand O-dealkylation of anisoles and ethoxycoumarins apparently proceeds via an α-hydroxylation mechanism (Watanabe et al., 1982; Miwa et al., 1984). We initially turned our attention to the ester (4-benzoyloxy)acetic acid, which is the ester cognate of the amide substrate 4-methoxyhippuric acid, anticipating that the ester might represent a potent PAM inhibitor. Indeed assays with **4** indicated that this ester is not a substrate for PAM, and that it competitively inhibits the turnover of TNP-D-Tyr-Val-Gly. In contrast to these results with the ester, the ether analogue **7** is an excellent substrate by comparison to the other non-peptide substrates presented here. One possibility is that the difference between the ester and the ether analogues reflects small differences in binding, which allow an α-hydroxylation pathway to be operative in the case of the ether, but which result in the α-protons of the ester being inaccessible to the copper–oxygen species. In this regard, we note that in the case of DBM, where enzyme specificity precludes an initial α-hydroxylation pathway, ether cognates are indeed potent competititve inhibitors and do not undergo O-dealkylation (Wimalasena & May, 1987).

Sulfoxidation is a facile monooxygenase reaction, and our laboratory has demonstrated sulfoxidation by both DBM (May & Phillips, 1980) and *Pseudomonas oleovorans* monooxygenase, a non-heme iron enzyme (Katopodis et al., 1988). Several other enzymes, including P_{450} (Waxman et al., 1982) and flavin monooxygenase (Light et al., 1982), also catalyze sulfoxidation. The results reported here establish that the sulfide **5** is an excellent substrate for PAM, and the V_{max} for sulfoxidation is comparable to that of TNP-D-Tyr-Val-Gly. An interesting aspect of the PAM reaction with **5** is the small amount of S-dealkylation observed. Since the sulfur cation radical has a strong tendency toward sulfoxidation (Guengerich et al., 1982; Guengerich & MacDonald, 1984), it seems probable that the competing pathway for S-dealkylation entails initial oxygenative attack at the α-carbon leading to an α-hydroxy sulfide, which then readily breaks down to the thiol and glyoxylate.

The reaction of PAM with amine **6** is directly analogous to the amidation reaction. The obvious difference between amine and amide PAM substrates is that, at physiological pH, alkylamines are protonated and this may lead to poor binding at the active site and poor reactivity toward the electrophilic copper–oxygen-activated species. Consistent with this notion, we find that the reactivity of the amine substrate has a pH maximum higher than our other substrates.

The reaction of PAM with olefin **8** leads to turnover-dependent inactivation of the enzyme. Monooxygenase reactions with olefins are well-known both to produce epoxide products and to lead to enzyme inactivation. Because no epoxide product was detected, and because incubations of PAM with authentic epoxide did not lead to inactivation, we suggest that an intermediate along the olefin oxygenation pathway is the actual inactivating species. This is also the case in the inactivation of both DBM (Padgette et al., 1985) and P_{450} (Ortiz de Montellano et al., 1982) by olefinic substrate analogues, where it has been demonstrated that it is not the epoxide but

rather an intermediate along the olefin oxygenation pathway which is responsible for turnover-dependent enzyme inactivation.

A number of mechanistic possibilities can be visualized for PAM-catalyzed oxygenative amidation. These range from direct α-hydroxylation, analogous to the mechanism that is apparently operative for P_{450}-catalyzed O-dealkylations, to mechanisms that involve initial generation of the nitrogen-centered cation radical. Mechanisms of the latter type are analogous to that operative in amine N-dealkylation reactions catalyzed by DBM (Wimalasena & May, 1987), P_{450} (Guengerich & MacDonald, 1984) and amine oxidase (Silverman et al., 1980), as well as to the mechanism of peroxidase-catalyzed oxidations of aniline and N- and ring-substituted anilines (Saunders & Mann, 1940; Naylor & Saunders, 1950). Such a mechanism has been postulated for both P_{450}-catalyzed (Shono et al., 1982) and anodic (Ross et al., 1975) amide N-dealkylations, which are directly analogous to the PAM reaction. It is also conceivable that an N-hydroxylamide intermediate may be formed along the pathway of PAM amidation. Such an intermediate is formed in the oxidation of hippuric acids by lead tetraacetate (Gledhill et al., 1986).

While the results reported here with novel small molecule substrates do not differentiate between the mechanistic possibilities, they do establish a multifaceted reactivity for this enzyme that parallels that of DBM and P_{450}. Moreover, our findings of O- and S-dealkylation by PAM represent good evidence that PAM is, at least in these cases, capable of carrying out α-hydroxylation. Finally, our finding of potent turnover-dependent PAM inhibition by olefins opens up new possibilities for mechanistic studies of this enzyme and for the design of inhibitors with possible pharmaceutical interest. Incorporation of olefin functionalities into peptide analogues may confer higher specificity and even tissue selectivity for the various forms of PAM.

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Saccharomyces cerevisiae Phosphoenolpyruvate Carboxykinase: Physicochemical Characteristics of the Nucleotide Binding Site, As Deduced from Fluorescent Spectroscopy Measurements†

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ABSTRACT: *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] is inactivated by the fluorescent sulfhydryl reagent *N*-(iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS). The inactivation reaction follows pseudo-first-order kinetics with respect to active enzyme to less than 10% remaining enzyme activity, with a second-order inactivation rate constant of 2.6 min^{-1} mM^{-1} at pH 7.5 and 30 °C. A stoichiometry of 1.05 mol of reagent incorporated per mole of enzyme subunit was found for the completely inactivated enzyme. Almost complete protection of the enzyme activity and of dansyl label incorporation are afforded by MnADP or MnATP, thus suggesting that 1,5-IAEDANS interacts with an enzyme sulfhydryl group at the nucleotide binding site. The fluorescence decay of the AEDANS attached to the protein shows a single-exponential behavior with a lifetime of 18 ns. A comparison of the fluorescence band position and the fluorescence decay with those of the adduct AEDANS-acetylcysteine indicates a reduced polarity for the microenvironment of the substrate binding site. The quenching of the AEDANS moiety in the protein can be described in terms of a collisional and a static component. The rate constant for the collisional component is much lower than that obtained for the adduct in a medium of reduced polarity. These last results indicate that the AEDANS moiety is considerably shielded from the solvent when it is covalently attached to PEPCK.

Yeast phosphoenolpyruvate carboxykinase (PEPCK)[1] [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] catalyzes the reaction

$$ATP + OAA \xrightleftharpoons{Mn^{2+}} PEP + ADP + CO_2 \quad (1)$$

which is an important regulatory step in the biosynthesis of glucose from C_3 and C_4 precursors (Utter & Kolenbrander, 1972; Gancedo & Schwerzman, 1976). Most animal PEPCKs are GTP-dependent enzymes composed of single polypeptide chains of about 70 kDa, whereas the yeast enzyme is an ATP-dependent tetramer with a subunit molecular mass of 61.4 kDa (Utter & Kolenbrander, 1972; Stucka et al., 1988). The presence of quaternary structure and a clear specificity for adenine nucleotides have also been reported for the carboxykinases isolated from C_4 plants (Burnell, 1986) and from *Trypanosoma cruzi* (Urbina, 1987).

It has been communicated that sulfhydryl-directed reagents inactivate PEPCKs from various sources (Utter & Kolenbrander, 1972), and the presence of essential sulfhydryl groups is well documented in the sheep kidney (Barns & Keech, 1972) and rat liver enzymes (Carlson et al., 1978; Lewis et al., 1989).

The complete amino acid sequences of the enzymes from rat liver, chicken kidney, *Drosophila melanogaster* heads, and

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[1] Abbreviations: 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; AEDANS, *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; PEP, phosphoenolpyruvate; OAA, oxalacetate; PEPCK, phosphoenolpyruvate carboxykinase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; P_i, inorganic orthophosphate.

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