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Thiorphan, tiopronin, and related analogs as substrates and inhibitors of peptidylglycine α-amidating monooxygenase (PAM)

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Abstract **Peptidyglycine α-amidating monooxygenase is a copper- and zinc-dependent, bifunctional enzyme that catalyzes the cleavage of glycine-extended peptides or *N*-acylglycines to the corresponding amides and glyoxylate. This reaction is a key step in the biosynthesis of bioactive α-amidated peptides and, perhaps, the primary fatty acids amides also. Two clinically useful *N*-acylglycines are thiorphan and tiopronin, each with a thiol moiety attached to the acyl group. We report here that thiorphan and tiopronin are substrates for PAM, exhibiting relatively low $K_{M,app}$ and $V_{MAX,app}$ values. The low $V_{MAX,app}$ values result, most likely, from a decrease in active PAM · 2Cu(II) as the enzyme competes ineffectively with thiorphan and tiopronin for free copper.**

Keywords: Peptidylglycine α-amidating enzyme; Enzyme inhibition; Tiopronin; Thiorphan

1. Introduction

Thiorphan (*N*-[(*R*,*S*)-3-mercapto-2-benzylpropionyl]glycine) and tiopronin (*N*-(2-mercaptopropionyl)glycine) or *N*-(2-mercapto-1-oxopropyl)glycine, also known as thiola are two thiol containing drugs. Thiorphan, administered as the prodrug racecadotril (also called acetorphan), is a low nanomolar inhibitor of neutral endopeptidase (NEP or enkephalinase, EC, 3.4.24.11) [1] and is used as an antidiarrheal [2,3]. Tiopronin is a metal chelator and antioxidant used to treat cystinuria [4], mercury and copper poisoning [5,6], and rheumatoid arthritis [7]. In addition, tiopronin is cytoprotective during myocardial ischemia and reperfusion [8,9] and may find use as a nephroprotective agent for the toxicity associated with cisplatin treatment [10].

Both thiorphan and tiopronin are *N*-substituted glycines (Fig. 1). Work from our laboratory [11–14] and from the May group [15] has shown that *N*-substituted glycines, the *N*-acyl- and *N*-arylglycines, are substrates for peptidylglycine α-amidating monooxygenase (PAM, EC 1.14.17.3). PAM catalyzes the final reaction in α-amidated peptide biosynthesis [16,17] and recent evidence suggests that the enzyme may also have a role in the biosynthesis of oleamide and other fatty acid primary amides [18,19]. PAM is a bifunctional enzyme consisting of separate catalytic domains. Peptidyl α-hydroxylating monooxygenase (PHM) catalyzes the copper-, O_2-, and ascorbate-dependent hydroxylation of the terminal glycyl α-carbon while peptidylamidoglycolate lyase (PAL) catalyzes the zinc-dependent dealkylation of the α-hydroxylglycine intermediate to the amide product and glyoxylate (Fig. 2) [20,21].

We report here that thiorphan, tiopronin, and number of structurally related analogs are substrates or inhibitors of PAM. Both thiophan and tiopronin bind to PAM with K_d values $\leqslant$100 μM, yet show low rates of amidation. The K_d values for thiorphan and tiopronin are equal to or lower than the $K_{M,app}$ values for the glycine-extended peptide substrates for PAM, which is surprising for such small, simple compounds. Generally, small structurally simple PAM substrates like *N*-benzoylglycine and short-chain *N*-acylglycines exhibit $K_{M,app}$ values >1.0 mM. Modeling studies indicate that the relatively strong affinity of tiopronin and thiorphan for PAM is a result of their interaction with copper and that the low rates of amidation observed for these compounds, most likely, results from a decrease in the PAM · 2Cu(II) concentration. PAM requires two bound copper atoms for maximum activity. This study provides new insights not only about the pharmacological activity of thiorphan and tiopronin, but also into the development of novel drugs targeted against PAM.

2. Materials and methods

2.1. Materials

α-Hydroxyhippuric acid, α-methylhydrocinnamic acid, isobutyric anhydride, and oxalyl chloride were from Aldrich, tiopronin, *S*-propylglutathione, and *N*-acetylglycine were from Sigma, thiorphan was from Bachem, *N*-dansyl-Tyr-Val-Gly was from Fluka BioChemika, and bovine catalase was from Worthington. Recombinant rat PAM was gift from Unigene Laboratories, Inc. All other reagents were of the highest quality available from commercial suppliers.

2.2. Synthesis

N-[(*R*,*S*)-2-Methylhydrocinnamoyl]glycine, *N*-[(*R*,*S*)-2-ethylhydrocinnamoyl]glycine, *N*-isobutyrylglycine, and *S*-methyltiopronin were prepared by conventional procedures and authenticated by elemental analysis (Atlantic Microlab Inc.) and ^{1}H NMR (Inova 400 spectrophotometer), $CDCl_3$–DMSO-d_6 (95:5) or D_2O, chemical shifts ppm (δ) relative to TMS or DSS. Full experimental details are provided below.

2.2.1. S-Methyltiopronin (N-(2-methylsulfanyl-1-oxopropyl)glycine). A solution of tiopronin (0.8 gm, 4.9 mmol) and sodium carbonate (0.6 gm, 5.7 mmol) in water (6 mL) was stirred with methyl iodide (0.8 gm, 5.6 mmol) until the organic layer disappeared and carbon dioxide evolution ceased (60 min), then acidified (3 M HCl, 4 mL) and extracted repeatedly with diethyl ether. Crude product (0.65 gm, 75%) recrystallized from ethyl acetate m.p. 109–110 °C [Ref. [22], 112–113 °C]. ^{1}H NMR (D_2O): δ 1.26 (d, J = 7 Hz, 3H), 1.99 (s, 3H), 3.37 (q, J = 7 Hz, 1H) 3.87 (s, 2H).

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Thiorphan
(*N*-[(*R,S*)-3-mercapto-2-benzylpropionyl]glycine)

Tiopronin
(*N*-(2-mercaptopropionyl)glycine)

Fig. 1. Thiorphan and tiopronin structures. The arrows indicate the bond cleaved by PAM.

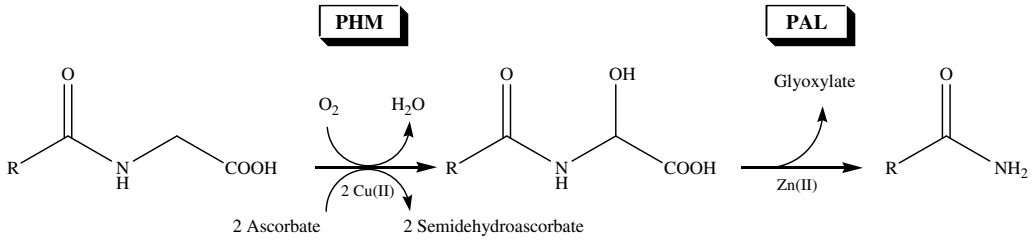

Fig. 2. The reaction catalyzed by bifunctional PAM. PAM is a comprised of two distinct catalytic entities: peptidyl α-hydroxylating monooxygenase (PHM) and peptidylamidoglycolate lyase (PAL).

2.2.2. N-[(R,S)-2-Ethylhydrocinnamoyl]glycine (N-(2-benzylbutyryl)-glycine). α-Ethylcinnamic acid [23] (3.5 gm, 20 mmol) in aqueous sodium hydroxide (22 mL 1 M) was stirred three days with sodium amalgam (sodium, 1.35 gm, 60 mg-at, in mercury, 100 gm). The filtered aqueous phase was acidified and extracted with dichloromethane to give α-ethylhydrocinnamic acid (3.5 gm, quantitative) as an oil, shown by NMR to be free from unsaturated precursor. This acid (2.6 gm, 15 mmol) reacted with oxalyl chloride (5.6 gm, 45 mmol) for 1 h at 25 °C then 15 min at 60 °C. The solvent was then stripped of volatiles and the resulting acid chloride was stirred overnight with a solution of glycine (1.7 gm, 23 mmol) and sodium carbonate (4.8 gm, 45 mmol) in water (30 mL). Acidification gave a precipitate of the target *N*-acylglycine. Recrystallized from diethyl ether (100 mL), 2.29 gm, 65%, m.p. 107–109 °C. ^{1}H NMR ($CDCl_3$): δ 0.85 (t, $J = 7$ Hz, 3H), 1.41–1.50 (m, 1H), 1.56–1.65 (m, 1H), 2.23–2.30 (m, 1H), 2.64 (dd, $J_1 = 6.5$ $J_2 = 13.5$ Hz, 1H), 2.87 (dd, $J_1 = 8.5$ $J_2 = 13$ Hz, 1H), 3.75 (dd, $J_1 = 4.5$ $J_2 = 19$ Hz,1H), 3.95 (dd, $J_1 = 5$ $J_2 = 19$ Hz, 1H), 6.16 (s, 1H), 7.01–7.20 (m, 5H).

2.2.3. N-[(R,S)-2-Methylhydrocinnamoyl]glycine (N-(2-benzylpropionyl)glycine). α-Methylhydrocinnamic acid (1.64 gm, 10 mmol) reacted with oxalyl chloride (2.5 gm, 20 mmol) for 1 h at 25 °C then 15 min at 60 °C. The solvent was stripped of volatiles and the resulting acid chloride stirred overnight with a solution of glycine (1.5 gm, 20 mmol) and sodium carbonate (4.1 gm, 40 mmol) in water (40 mL). Acidification gave a crystalline precipitate of the target *N*-acylglycine (1.55 gm, 70%). Recrystallized from diethyl ether (50 mL) mp 113–114 °C. ^{1}H NMR (CDCl3): δ 1.65 (d, $J = 7$ Hz, 3H), 2.51–2.68 (m, 2H), 3.01 (dd, $J_1 = 7$ $J_2 = 14$ Hz, 1H), 3.87(dd, $J_1 = 4.5$ $J_2 = 18.5$ Hz, 1H), 4.01 (dd, $J_1 = 4.5$ $J_2 = 18.5$ Hz, 1H), 6.20 (br.s, 1H), 7.15–7.29 (m, 5H).

2.2.4. N-Isobutyrylglycine (3-aza-4-oxo-5-methylhexanoic acid). Isobutyric anhydride (32 gm, 200 mmol) was added in one portion to a stirred mixture of glycine (7.5 gm, 100 mmol) and water (30 mL). The mixture was heated, eventually to boiling, then stripped of volatiles under reduced pressure to give a waxy solid which was extracted with 10 successive portions of boiling diethyl ether. Partial evaporation of the extracts gave the crystalline acylglycine (6.3 gm, 45%) m.p. 104–106 °C. ^{1}H NMR ($CDCl_3$): δ 1.17 (d, $J = 7$ Hz, 6H), 2.41–2.51 (m, 1H), 3.99 (s, 2H), 6.54 (br.s, 1H).

2.3. Molecular modeling

Glide (grid-based ligand docking with energetics) from the FirstDiscovery 3.0 suite (www.schrodinger.com) was used for substrate analysis [24,25]. The coordinates of oxidized and reduced peptidylglycine α-hydroxylating monooxygenase (PHM) were obtained from the Protein Data Bank [26,27]. The enzyme-bound coppers, Cu_M and Cu_H, were selected within *atom type parameters* to be Cu(I) for reduced and Cu(II) for oxidized PHM grids.

Several protein grids were prepared: reduced PHM · 2Cu(I) with bound O_2, reduced PHM · 2Cu(I) with no bound O_2, and oxidized PHM · 2Cu(II). Prior to determination of the grid enclosure, species determined to be redundant for ligand binding were removed (nickel, water, glycerol and co-crystallized substrate). With no further energy minimizations of the co- crystallized protein structure, *ProteinPrep* was used to add hydrogens and perform a restrained minimization. The *bounding* box containing all ligand atoms was increased from 10 Å^3 (default) to 14 Å^3. This was done to ensure all residues surrounding both PHM copper binding sites were included within the *enclosing* box. Substrates underwent energy minimization in Chem3D Ultra 8.0 with MM2 and were then imported into *Maestro*. *Standard Precision* mode was used for ligand docking investigation with 30–50 poses per ligand. The top pose for each ligand was selected based on the correlation of *Emodel* and *Glide* score, respectively. Positive controls were completed for each experiment through re-docking of the co-crystallized substrate.

2.4. Determination of $K_{M,app}$ and $V_{MAX,app}$ values for N-[(R,S)-2-methylhydrocinnamoyl]glycine, N-[(R,S)-2-ethylhydrocinnamoyl]glycine, L-alanylglycine, N-(isobutyryl)glycine, and S-methyltiopronin

Reactions at 37.0 ± 0.1 °C were initiated by the addition of PAM (30–70 μg) into 2.0–3.0 mL of 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0 μM $Cu(NO_3)_2$, 5.0 mM sodium ascorbate, and the oxidizable substrate (generally 0.3–5.0 $K_{M,app}$). Initial rates were measured by following the PAM-dependent consumption of O_2 using a Yellow Springs Instrument Model 53 oxygen monitor interfaced with a personal computer using a Dataq Instruments analogue/digital converter (model DI-154RS). $V_{MAX,app}$ values were normalized to controls performed at 11.0 mM *N*-acetylglycine. Ethanol was added to protect the catalase against ascorbate-mediated inactivation and Triton X-100 was included to prevent non-specific absorption of PAM to the sides of the oxygen monitor chambers.

2.5. Inhibition of O_2 consumption from N-acetylglycine by tiopronin

Reactions at 37.0 ± 0.1 °C were initiated by the addition of PAM (36 μg) into 2.5 mL of 100 mM MES/NaOH, pH 6.0, 30 mM NaCl, 5.0 mM sodium ascorbate, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 μg/ml bovine catalase, 1.0 μM $Cu(NO_3)_2$, 2.5–20 mM