Pyrurate-extended Amino Acid Derivatives as Highly Potent Inhibitors of Carboxyl-terminal Peptide Amidation*

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Carboxyl-terminal amidation, a required post-translational modification for the bioactivation of many neuroopeptides, entails sequential enzymatic action by peptidylglycine monooxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5). The monooxygenase, PAM, first catalyzes conversion of a glycine-extended pro-peptide to the corresponding α-hydroxyglycine derivative, and the lyase, PGL, then catalyzes breakdown of this α-hydroxyglycine derivative to the amidated peptide plus glyoxylate. We now introduce the first potent inhibitors for peptidylamidoglycolate lyase. These inhibitors, which can be viewed as pyruvate-extended N-acetyl amino acids, constitute a novel class of compounds. They were designed to resemble likely transient species along the reaction pathway of PGL catalysis. A general synthetic procedure for preparation of pyruvate-extended N-acetyl amino acids or peptides is described. Since these compounds possess the 2,4-dioxo-carboxylate moiety, their solution tautomerization was investigated using both NMR and high performance liquid chromatography analyses. The results establish that freshly prepared solutions of N-Ac-Phe-pyruvate consist predominantly of the enol tautomer, which then slowly tautomerizes to the diketo form when left standing for several days in an aqueous medium; upon acidification, formation of the hydrate tautomer occurs. Kinetic experiments established that these novel compounds are highly potent, pure competitive inhibitors of PGL. Kinetic experiments with the ascorbate-dependent copper monoxygenases, PAM and dopamine-β-monooxygenase, established that these compounds also bind competitively with respect to ascorbate; however, pyruvate-extended N-acetyl amino acid derivatives possessing hydrophobic side chains are much more potent inhibitors of PGL than of PAM. Selective targeting of N-Ac-Phe-pyruvate so as to inhibit the lyase, but not the monoxygenase, domain was demonstrated with the bifunctional amidating enzyme of Xenopus laevis. The availability of potent inhibitors of PGL should facilitate studies regarding the possible biological role of α-hydroxyglycine-extended peptides.

Neuropeptides, which are critical mediators of intercellular communication, are generated biosynthetically from larger precursors via a variety of post-translational modifications. One such processing event is carboxyl-terminal amidation, a very prevalent post-translational modification essential to the bioactivity of many neuopeptides (1, 2). We and others (3–8) have demonstrated that formation of peptide amides from their glycine-extended precursors is a two-step process, entailing sequential enzymatic action by peptidylglycine monooxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5). The monooxygenase, PAM, first catalyzes formation of the α-hydroxyglycine derivative of the glycine-extended precursor, in a process dependent upon ascorbate, copper, and molecular oxygen (3, 4, 9). The lyase, PGL, then catalyzes the breakdown of this α-hydroxyglycine derivative to produce the amidated peptide plus glyoxylate (3, 8).

We have established that PAM and PGL exhibit tandem stereospecificities in carrying out the two requisite steps of carboxyl-terminal amidation (10), with PAM producing exclusively α-hydroxyglycine moieties of absolute configuration (S), and PGL being reactive only toward (S)-α-hydroxyglycines (Scheme I). We further demonstrated that despite their tandem reaction stereospecificities, PAM and PGL exhibit sharply contrasting subsite stereospecificities toward the residue at the penultimate position (the P₂ residue) of their respective substrates and inhibitors (11). PAM exhibits high S₂ subsite stereospecificity, and both substrate reactivity and inhibitor binding are severely diminished by the presence of a D-amino acid residue (or of the stereotopically equivalent (R)-manderyl residue) at the P₂ position. In contrast, the configuration of the chiral moiety at the P₂ position of substrates or inhibitors of PGL has virtually no effect on binding or catalysis; the lyase thus exhibits concomitant high reaction stereospecificity and low S₂ subsite stereospecificity.

Amidation represents a potentially attractive target point for modulating the bioactivity of peptide hormones; consequently, a number of inhibitors for amidating enzymes have recently been reported. These include substrate-analog competitive inhibitors, i.e. O-glycolate esters (11, 13) and homocysteine-terminating peptides (14), as well as olefinic mechanism-based inactivators (13, 15, 16). All of these compounds are inhibitors of the PAM-catalyzed monooxygenation step in the amidation process. To date, the only report of PGL inhibition is our recent finding that the α-hydroxyglycine derivatives of phenylacetic acid (and its benzylallyl alkylation analogs) undergo such slow catalytic turnover that they behave kinetically as competitive inhibitors in PGL assays (11). Yet, the availability of potent inhibitors for the lyase step is essential for exploring the very

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1 The abbreviations used are: PAM, peptidylglycine α-monooxygenase; PGL, peptidylamidoglycolate lyase; MES-Na, sodium 2-(N-morpholinol)ethanesulfonate; TNP, trinitrophenyl; HPLC, high performance liquid chromatography; DBM, dopamine β-monooxygenase; IFN, interferon; FAB, fast atom bombardment; CI, chemical ionization; MS, mass spectrometry.

2 The nomenclature for amino acid residues (or analogs) of substrates (P₁, P₂, etc.) and the corresponding enzyme subsites (S₁, S₂, etc.) is that originally proposed by Schecter and Berger (12).
Intriguing question of whether the α-hydroxyglycinnated peptides generated in the amidation process may exhibit distinct biological activities (3, 8).

We introduce here the first potent inhibitors for pyrrolidinodiglyceroylase. These inhibitors, which are 2,4-diketo-5-acetamido-alkanoic acid derivatives but can be more conveniently viewed as amino acid derivatives which have been pyruvate-extended at the carboxyl terminus, constitute a novel class of compounds. They were designed to resemble likely transient species along the reaction pathway of PGL catalysis. We report a general synthetic procedure for preparation of pyruvate-extended N-acetyl amino acids or peptides; we describe their solution tautomization on the basis of NMR and HPLC analyses; and we characterize kinetically their interaction with both PGL and PAM. In addition, we demonstrate targeting of these inhibitors so as to selectively inhibit the lyase, but not the monoxygenase, domain of a bifunctional amidating enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

Frozen bovine pituitaries were purchased from Pel Freeze Biologicals (Rogers, AR). Diethyl oxalate was obtained from Aldrich Chemical Co. Ac-Tyr-Val-Gly (Ac-YVG) was from Peninsula Laboratories, Inc. Bovine liver catalase was from Boehringer Mannheim. Ascorbic acid, all buffers, and amino acids were from Sigma; α-Tyr-Val-Gly (TVG) was from Bachem (Torrance, CA).

**Methods**

**Enzyme Purification and Assays—**PAM and PGL were isolated from bovine pituitaries as described previously (3). PAM and PGL assays were performed in 100 mM MES- Na buffer, pH 6.5, at 37°C. PAM assays contained 4 mM ascorbic acid, 4 μM CuSO4, and 1 mg/ml catalase, as described previously (3). The bifunctional amidating enzyme from Xenopus laevis skin (17) was expressed using the Spodoptera frugiperda baculovirus expression system followed by sequential column chromatography (SP Sepharose, Superose 12) using a Pharmacia FPLC (fast protein liquid chromatography) system. PAM and PGL inhibition was established using TNP-α-Tyr-Val-Gly and TNP-α-Tyr-Val-α-hydroxy-Gly as the PAM and PGL substrate, respectively. Product formation was analyzed quantitatively by HPLC using a standard curve based on peak height. All PGL rates measured were corrected for a background rate observed in the absence of enzyme. HPLC analyses were performed on a Spherisorb C8 reversed-phase column (Alltech), using an LDC Constatmic III system outfitted with an LDC Spectrometer 3100 variable wavelength detector set at 300 nm. The eluting solvent was 20% (v/v) acetonitrile, 79.9% water, and 0.1% trifluoroacetic acid. 1H NMR spectra were recorded on a Bruker 300 MHz spectrometer.

**Syntheses**

**Benzoylpyruvic Acid—**Acetophenone (25.0 g, 0.21 mol) and diethyl oxalate (25.5 ml, 0.19 mol) were added to a mixture of sodium ethoxide (17.0 g, 0.25 mol) in 250 ml of super-dry ethanol, under argon (20). The reaction mixture was stirred at room temperature for 4 h. Ethanol was then evaporated under reduced pressure and water added to the residue. The aqueous layer was extracted with ethyl diethyl oxalate, acidified by the addition of concentrated HCl, and extracted with methylene chloride. The combined methylene chloride extracts were dried over MgSO4, filtered, and concentrated under reduced pressure. Ethyl benzoylpyruvate, obtained as an oil, was not further purified. 1H NMR (CDCl3, 300 MHz): δ 8.00 (d, 2H), 7.65–7.49 (m, 3H), 7.09 (s, 1H), 4.41 (q, 2H), 1.42 (t, 3H).

A solution of ethyl benzyloxypyrurate (47.4 g of crude ester) in 125 ml of THF was added to 125 ml of concentrated HCl. The reaction mixture was stirred at room temperature for 48 h and then cooled to 0°C to maximize the precipitation of the acid. The solid that formed was then collected by suction, washed with cold THF, and dried under vacuum. Benzoylpyruvic acid (37.0 g) was obtained as a light yellow powder. Mass spectrometry (electron impact-MS) m/z 192 (M), 1H NMR (CDCl3, 300 MHz): δ 8.01 (d, 2H), 7.65 (m, 1H), 7.53 (m, 2H), 7.19 (s, 1H), 4.70 (t, 2H), 4.31 (q, 2H), 3.02–3.18 (m, 2H), 1.99 (s, 3H), 1.32 (t, 3H).

**Ac-Phe-Pyruvate (2,4-Diketo-5-acetamido-6-phenyl-hexanoic Acid)—**A solution of L-phenylalanine (33.0 g, 0.2 mol) in acetic anhydride (165 ml) and pyridine (110 ml) was stirred at 100°C for 5 h, under argon (21). The cooled reaction mixture was then concentrated under reduced pressure. The residue was dissolved in ethyl acetate, washed three times with saturated sodium bicarbonate solution, dried over MgSO4, filtered, and concentrated. The residue was re-crystallized from hot ethyl acetate. The white α-acetamidobenzyl methyl ketone was filtered off, washed with hexane, and dried under vacuum. Yields ranged from 30 to 70%, m.p. 90–95°C; mass spectrometry (CI-MS) m/z 306 [M + 1]; 1H NMR (CDCl3, 300 MHz): δ 7.30 (m, 5H), 7.14 (m, 2H), 6.11 (bs, 1H), 4.88 (q, 1H), 3.02–3.18 (m, 2H), 2.16 (s, 3H), 1.99 (s, 3H).

**Calculation:** C70.22H7.37N6.82 Found: C70.15 H 7.38 N 6.89

Sodium ethoxide was prepared in situ by the addition, under argon, of sodium metal (0.92 g, 40.0 mmol) in 50 ml of super-dry ethanol kept at 0°C. This solution was added α-acetamidobenzyl methyl ketone (4.44 g, 20 mmol) and diethyl oxalate (11.0 ml, 81.0 mmol). The reaction mixture was stirred at room temperature, under argon, for 4 h. Ethanol was then evaporated under reduced pressure and water added to the residue. The aqueous layer was extracted with ethyl acetate, acidified by the addition of concentrated HCl, and extracted with methylene chloride. The combined methylene chloride extracts were dried over MgSO4, filtered, and concentrated under reduced pressure. Purification by flash chromatography (eluting solvent: MeCl/MeOH 95:5, Rf = 0.49) gave 5.40 g (88%) of a thick brown oil. Mass spectrometry (CI-MS) m/z 306 (M + 1); 1H NMR (CDCl3, 300 MHz): δ 7.26 (m, 3H), 7.06 (m, 2H), 5.98 (bd, 1H), 4.95 (q, 1H), 4.31 (q, 2H), 3.02–3.18 (m, 2H), 1.99 (s, 3H), 1.32 (t, 3H).

A solution of ethyl-N-Ac-Phe-pyruvate (3.66 g, 12.0 mmol) in 20 ml methanol was added 10 ml of 4 N NaOH. The reaction mixture was stirred at room temperature for 2 h, under argon. Methanol was then removed under reduced pressure and 20 ml water added. The aqueous solution was extracted with ethyl acetate to remove any un-reacted ester, acidified by the addition of concentrated HCl, and extracted with methylene chloride. The combined methylene chloride extracts were dried over Na2SO4 and concentrated under reduced pressure. The resulting free acid (2.1 g, 7.57 mmol) was dissolved in 30 ml of methanol.
and converted to its sodium salt by addition of NaOH (304.0 mg, 7.60 mmol). The reaction mixture was stirred at room temperature for 2 h and ether added to precipitate the desired compound. The precipitate was collected by suction, dissolved in methanol, and precipitated again by the addition of ether, affording 1.6 g of pale yellow solid. Mass spectrometry (FAB-MS) m/z 266 (M + 1); ¹H NMR (CDCl₃, 300 MHz) δ: 7.20 (m, 5H), 3.10, 2.75 (m, 2H), 1.82 (s, 3H).

Ac-Leu-Pyruvate (2,4-Diketo-5-acetamido-7-6-(4'-hydroxy-phenyl)-hexanoic Acid)—The Dakin-West reaction was performed as described for Ac-Phe-pyruvate, starting from L-tyrosine (18.1 g, 68.74 mmol). The free acid (5.26 g, 0.10 mol) was treated with sodium ethoxide in super-dry ethanol to give a pale yellow oil (16.1 g, 89%) of white solid. m.p. 117–119°C; mass spectrometry (CI-MS) m/z 278 (M + 1); ¹H NMR (CDCl₃, 300 MHz) δ: 7.12 (d, 2H), 6.71 (d, 2H), 3.00, 2.61 (m, 2H), 1.80 (s, 3H).

Ac-Met-Pyruvate [2,4-Diketo-5-acetamido-7-thiomethyl-heptanoic Acid]—The Dakin-West reaction was performed as described for Ac-Phe-pyruvate, starting from DL-methionine (14.9 g, 0.10 mol). Purification by flash chromatography (eluting solvent: MeCl₂/MeOH 50:1) gave 10.3 g (63%) of a pale yellow oil. Mass spectrometry (CI-MS) m/z 293 (M + 1); ¹H NMR (CDCl₃, 300 MHz) δ: 7.12 (d, 2H), 6.71 (d, 2H), 3.00, 2.61 (m, 2H), 1.80 (s, 3H).

RESULTS AND DISCUSSION

Synthesis of 2,4-Diketo-5-acetamido-alkanoic Acids—Since the pyruvate-extended amino acid derivatives reported here are novel compounds, several synthetic approaches were explored. We found that our initial prototype, benzoylpyruvate, could be prepared in high yield by reaction of acetophenone with diethylxylalate in the presence of NaOEt to give ethyl benzoylpyruvate (20), followed by hydrolysis of this ester under acidic conditions. We therefore employed the synthetic sequence illustrated in Scheme II for the preparation of N-Ac-Phe-pyruvate (2,4-diketo-5-acetamido-6-phenyl-hexanoic acid), N-Ac-Leu-pyruvate (2,4-diketo-5-acetamido-7-methyl-octanoic acid), N-Ac-Tyr-pyruvate (2,4-diketo-5-acetamido-6-(4'-hydroxy-phenyl)-hexanoic acid), and N-Ac-Met-pyruvate (2,4-diketo-5-acetamido-7-thiomethyl-heptanoic acid). The parent amino acids, Phe, Leu, Tyr, and Met, obviously represent convenient starting materials. In order to apply the synthetic strategy used for benzoylpyruvate, the carboxylic acid functionality had to be first converted to a ketone. The first step in Scheme II thus entails conversion of the amino acids 1a–d to the corresponding α-acetamido-methyl ketones 2a–d via the Dakin-West reaction (21); in our hands, ketone yields ranged from 50 to 90%. Reaction with sodium ethoxide in super dry ethanol followed by condensation with diethyl oxalate then gave the desired esters, 3a–d. Finally, hydrolysis of the esters was carried out under basic conditions, since we found that acidic hydrolysis, as had been done successfully for benzoylpyruvate, led to decomposition of the desired compounds. The final compounds, 4a–d, were isolated as the sodium salts; typical overall yields after isolation and final purification were 20–30%.

Keto-Enol Tautomerism—As illustrated in Scheme III, 2,4-dioxo-carboxylic acids and esters have been reported to exist in three tautomeric forms (22, 23): enol (i.e. two possible enols, E1 and E2), diketo (K), and hydrate (H), with the equilibrium distribution between these tautomers being dependent on pH and solvent. Fig. 1 shows reverse phase HPLC chromatograms of a solution of N-Ac-Phe-pyruvate in water; chromatograms of the freshly prepared, 1- and 4-day-old solutions are shown in Fig. 1, A–C, respectively. The relative intensities of the peaks
at 6.2 and 10.3 min change with time, such that after 4 days the peak at 10.3 min has almost disappeared while that at 6.2 min has increased about 80-fold. This process is accelerated by heating. When the pH of the solution is lowered, a prominent new peak appears at 16.3 min (Fig. 1D), which increases with time while the peaks at 6.2 and 10.3 min disappear. Thus, these HPLC results indicate that N-Ac-Phe-pyruvate exists as an equilibrium distribution of three tautomeric forms, the ratio of each being dependent on the pH of the solution. The solution effects suggest assignment of the peaks at 6.2, 10.3, and 16.3 min to the keto, enol (the two enol forms being indistinguishable under these conditions), and hydrate tautomers, respectively, with the appearance of the hydrate only under acidic conditions being consistent with studies by Becker and co-workers (24, 25) on the hydration of pyruvate.

The tautomeration of N-Ac-Phe-pyruvate was then investigated using 1H NMR. As shown in Fig. 2A, a fresh solution of N-Ac-Phe-pyruvate in D$_2$O exhibits a singlet at 1.90 ppm corresponding to the N-acetyl methyl group, a multiplet at 2.75–3.26 ppm typical of the benzylic methylene group of phenylalanine and its derivatives, and a multiplet centered around 7.30 ppm for the phenyl ring. The methyne resonance of the phenylalanine moiety is masked by the HOD peak at 4.8 ppm. After 2 days at room temperature, two distinct singlets at 1.90 and 1.95 ppm can be seen, accompanied by an enhancement of the peak at 2.39 ppm (Fig. 2B, panel II versus panel I). After 20 days, the peak at 2.39 ppm is prominent, and the singlet at 1.95 ppm is now more intense than the one at 1.90 ppm (Fig. 2B, panel III). After 30 days, the peak at 1.90 ppm has completely disappeared, and the NMR spectrum in the 1.8–2.4 ppm region consists of one singlet at 1.95 ppm and one singlet at 2.39 ppm, corresponding upon integration to three protons and two protons, respectively. The peak at 2.39 ppm is in the region where the resonance of the $\beta$-methylene of the diketo form is expected.
Thus, the positions and integrations of the 1.95 and 2.39 ppm peaks indicate that after 30 days complete conversion of the compound to the diketo tautomer has occurred. It is evident that this tautomerization to the keto form gives rise to the shift of the methyl signal from 1.90 to 1.95 ppm. Direct precedence for this interpretation is provided by the case of acetopyruvic acid, where a similar difference between the methyl signals of the tautomers has been reported (22).

Further support for these conclusions was obtained from NMR studies of N-Ac-Phe-pyruvate in dimethyl sulfoxide-$d_6$, wherein broad singlets are evident at 4.33 (methylene), 5.98, and 7.92 ppm (amide) in addition to the methyl signal at 1.71 ppm, the benzylic methylene at 2.60–3.02 ppm, and the phenyl resonance centered around 7.15 ppm. The singlet at 5.98 ppm is indicative of an olefinic-type proton. A converse experiment was then carried out wherein an aqueous solution of N-Ac-Phe-pyruvate was left standing for a week, lyophilized, and dissolved in dimethyl sulfoxide-$d_6$. The $^1$H NMR spectrum exhibited sharp signals and a singlet at 2.25 ppm, confirming that the compound had tautomerized to the diketo tautomer. Therefore, on the basis of these HPLC and NMR results, we conclude that a freshly prepared solution of N-Ac-Phe-pyruvate consists predominantly of the enol tautomer, characterized by a retention time of 10.3 min under our HPLC conditions. The compound then slowly tautomerizes to the diketo form when left standing in an aqueous medium.

**Inhibition of Peptidylamidoglycolate Lyase**—The rationale underlying our preparation and characterization of 2,4-diketo-5-acetamido-alkanoic acids as potential PGL inhibitors is based on our view of the likely reaction pathway for PGL catalysis. Ketonization at the α-hydroxyl with electron delocalization into the adjacent amido moiety, as illustrated in Scheme IV, pro-
vides a mechanistic driving force for the C—N bond cleavage which occurs in the course of the lyase reaction. Indeed, good precedence for such a reaction pathway is provided by the well established breakdown of the reactive indolene species which occurs in the mechanism of action of tryptophan-indole lyase (26, 27). Clearly, the pyruvate-extended N-acetyl-amino acid derivatives (which exist in solution in tautomeric enol and keto forms, as established by the HPLC and NMR results presented above) possess structural features which closely mimic the chemistry occurring along this reaction pathway for PGL catalysis. Since these pyruvate-extended N-acetyl-amino acids obviously cannot undergo PGL-catalyzed N-dealkylation, we expected that these compounds would act as potent inhibitors for PGL.

Initial kinetic experiments carried out using N-Ac-Phe-pyruvate confirmed that this compound is indeed a potent PGL inhibitor and that inhibition is purely competitive (Fig. 3). Corresponding experiments were then carried out with the other pyruvate-extended N-acetyl-amino acid derivatives, and the results are listed in Table I. In all cases, inhibition is purely competitive. It is apparent from the $K_I$ values that the hydrophobic aromatic side chain of a Phe residue at the S2 subsite favors binding to PGL. Accordingly, N-Ac-Tyr-pyruvate and N-Ac-Leu-pyruvate are somewhat less potent, whereas N-Ac-Met-pyruvate is more than 60-fold less potent than N-Ac-Phe-pyruvate.

**Effect of Pyruvate-extended N-Ac-Amino Acid Derivatives on Ascorbate Binding**—Since a dioxo-carboxylic acid moiety is present in these pyruvate-extended N-acetyl-amino acids, it seemed possible that these compounds might bear sufficient resemblance to ascorbate so as to interact with ascorbate binding sites of enzymes. Kinetic experiments to investigate this possibility were therefore carried out using both PAM and dopamine-β-monooxygenase (DBM); both of these enzymes are ascorbate-dependent copper monooxygenases, and we have previously noted that they have a number of mechanistic features in common (13, 28).

Inhibition experiments with DBM were carried out by monitoring oxygenation of the standard DBM substrate, tyramine, using a polarographic assay method (19). The results confirmed that N-Ac-Phe-pyruvate is a DBM inhibitor which is competitive with respect to ascorbate and uncompetitive with respect to tyramine. These findings are in accord with those reported by Townes et al. (29) with quinolinicarboxylic acids, picolinic acids, and thiazoline carboxylate; all of these compounds are DBM inhibitors which are competitive with respect to ascorbate and uncompetitive with respect to tyramine. Similarly, for PAM, inhibition by N-Ac-Phe-pyruvate was found to be competitive with respect to ascorbate and uncompetitive with respect to the peptide substrate, TNP-n-Tyr-Val-Val-Gly. Corresponding inhibition experiments with the other pyruvate-extended N-acetyl-amino acid derivatives also gave competitive inhibition patterns with respect to two different peptide substrates. Thus, these results indicate that the pyruvate-extended N-acetyl-amino acids interact in a kinetically similar way with these two analogous monooxygenases.

**Selective Targeting of the Lyase Domain of a Bifunctional Amidating Enzyme**—The kinetic inhibition results described above established that those pyruvate-extended N-acetyl-amino acid derivatives possessing hydrophobic side chains are much more potent inhibitors of PGL than of PAM. Thus, for example, the $K_I$ of N-Ac-Phe-pyruvate toward PGL is more than 2 orders of magnitude lower than that toward PAM, and the corresponding $K_I$ ratios for N-Ac-Tyr-pyruvate and N-Ac-Leu-pyruvate are 83 and 60, respectively. Accordingly, we utilized the bifunctional amidating enzyme from *X. laevis* skin (17) to demon-
strate selective targeting of the lyase, but not the monoxygenase domain, by N-Ac-Phe-pyrurate.

Fig. 4A shows the normal time course of the reaction of the substrate TNP-D-Tyr-Val-Gly with the bifunctional amidating enzyme; formation of each enzymatic product was quantita-
tively determined using reverse phase HPLC. Time-dependent formation of the monoxygenase product, TNP-n-Tyr-Val-α-OH-Gly, and, after an initial lag, of the final amide product, TNP-n-Tyr-Val-NH₂, is evident. The time course of this reaction in the presence of N-Ac-Phe-pyrurate (1.1 μM) is shown in Fig. 4B. It is clearly apparent that the lyase activity of the bifunctional enzyme has been abolished by the inhibitor, whereas the monoxygenase activity of the enzyme is completely unaffected. Thus, the initial rate of formation of the monoxygenase product, TNP-n-Tyr-Val-α-OH-Gly, is identical (0.25 μM/min) in either the presence (Fig. 4B) or absence (Fig. 4A) of N-Ac-Phe-pyrurate. In the absence of N-Ac-Phe-pyrurate, the concentration of the TNP-n-Tyr-Val-α-OH-Gly intermediate levels off at approximately 10 μM, since it is further converted by the lyase domain to the final amide product, TNP-n-Tyr-Val-NH₂. In contrast, in the presence of N-Ac-Phe-pyrurate, formation and accumulation of TNP-n-Tyr-Val-α-OH-Gly continues over the entire time course of the reaction, since lyase-catalyzed amide formation cannot occur. Moreover, it is important to note that the total amount of TNP-n-Tyr-Val-α-OH-Gly formed after 120 min (23 μM) in Fig. 4B corresponds to the sum of TNP-n-Tyr-Val-α-OH-Gly plus TNP-n-Tyr-Val-NH₂ formed (24 μM) in Fig. 4A. This confirms that catalytic turnover at the monoxygenase domain is completely unaffected by the presence of N-Ac-Phe-pyrurate.

These results clearly demonstrate selective inhibition by N-Ac-Phe-pyrurate of only lyase, and not monoxygenase, catalysis, under conditions where both PAM and PGL are present and functional. Physiologically, PAM and PGL are co-localized within secretory granules which maintain an acidic pH of approximately 5.5–6.0 (30). In this pH range, both PAM and PGL are highly active, whereas the rate of nonenzymatic α-hydroxyglycine peptide breakdown is expected to be quite low (8, 31). In this regard, we have carried out a kinetic study of the nonenzymatic N-dealkylation of a series of N-acetyl-α-hydroxyglycine compounds over the pH range 5.0–9.0. In all cases, these nonenzymatic reactions were found to be second order, with rate = k [N-acetyl-α-hydroxyglycine] [OH⁻]. In addition, each reaction product was confirmed to be the corresponding amide, using reverse phase HPLC analysis. The results are summarized in Table II. It is quite evident that all of the hydroxyglycines are quite stable under acidic conditions, with half-lives of weeks. Even when the pH is raised to 7.0, these compounds exhibit half-lives of several days. Similarly, the nonenzymatic half-life of our standard PGL assay substrate, TNP-n-Tyr-Val-α-OH-Gly, is more than 3 days. Thus, these results provide support for the expectation that amide-dissimulating processes of glycinextended peptides in the presence of a lyase inhibitor could well lead to a physiologically significant accumulation of α-hydroxyglycine-extended neuropeptides within secretory granules.

Carboxyl-terminal amidation has commonly been viewed as an activating event in neuropeptide biosynthesis (1, 2), with relatively little attention being given to possible biological roles of the glycine-extended precursors. In this regard, recent findings regarding the bioactivities of the amidated peptide, gastrin17-NH₂, and its glycine-extended analog are remarkable. Yamada et al. (32) have now shown that both gastrin17-NH₂ and gastrin17-Gly stimulate DNA synthesis in the exocrine pancreatic AR4–2J tumor cell line via distinct receptors (32). Moreover, they have also reported that gastrin17-NH₂ and gastrin17-Gly act cooperatively via different intracellular mechanisms to stimulate cell growth (33). These findings raise the highly intriguing possibility that, in addition to its role in bioactivation, carboxyl-terminal amidation may represent a mechanism for shifting the bioactivity of a given peptide hormone from one target to another. Obviously, key issues in this regard are the metabolic and regulatory relationships between glycine-extended peptides and the α-hydroxyglycinated peptides generated from them, as well as the important question of whether such hydroxyglycinated peptides which, as we have established, have substantial half-lives, exhibit distinct biological activities. The availability of potent lyase inhibitors should greatly facilitate studies regarding these important aspects of neuropeptide processing.

TABLE II

| Acyl-α-hydroxyglycine | Product | Half-life a | pH b |
|-----------------------|---------|------------|------|
| Phenylacetyl-α-hydroxyl-Gly | Phenylacetamide | 400 | 6.0 | 7.0 | 8.0 | 9.0 |
| 4-Methoxyphenylacetyl-α-hydro- Gly | 4-Methoxyphenylacetamide | 480 | 6.0 | 7.0 | 8.0 | 9.0 |
| 4-Nitrophenylacetyl-α-hydroxy-Gly | 4-Nitrophenylacetamide | 260 | 6.0 | 7.0 | 8.0 | 9.0 |
| Benzoyl-α-hydroxy-Gly | Benzaclidide | 240 | 6.0 | 7.0 | 8.0 | 9.0 |
| Cinnamoyl-α-hydroxy-Gly | Cinnamidide | 290 | 6.0 | 7.0 | 8.0 | 9.0 |
| 1-Naphthylacetyl-α-hydroxy-Gly | 1-Naphthylacetylglycine | 420 | 6.0 | 7.0 | 8.0 | 9.0 |

a Average S.E. values of 5% or less were calculated.

b pH values.
Inhibitors of Carboxyl-terminal Peptide Amidation

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