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5-Oxoprolinal: Transition-State Aldehyde Inhibitor of Pyroglutamyl-Peptide Hydrolase†

Theodore C. Friedman, Toni B. Kline, and Sherwin Wilk*
Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029
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ABSTRACT: Pyroglutamyl-peptide hydrolase (EC 3.4.11.8) removes the N-terminal pyroglutamyl residue from pyroglutamyl-containing peptides such as thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LH-RH), neurotensin, and bombesin. The aldehyde analogue of pyroglutamate, 5-oxoprolinal, was synthesized as an active site directed transition-state inhibitor of the enzyme. 5-Oxoprolinal was found to be a potent (K_i = 26 nM) and specific competitive inhibitor of pyroglutamyl-peptide hydrolase. Other aldehydes tested inhibited the enzyme only weakly or not at all. 5-Oxoprolinal blocked the degradation of LH-RH by purified pyroglutamyl-peptide hydrolase. The inhibitor, when injected into mice, inhibited the enzyme after 10 and 30 min. 5-Oxoprolinal should be of value in studies probing the biological significance of pyroglutamyl-peptide hydrolase.

Pyroglutamyl-peptide hydrolase (EC 3.4.11.8), an enzyme classified as a thiol protease, cleaves the N-terminal pyroglutamyl residue from pyroglutamyl-containing peptides such as thyrotropin-releasing hormone (TRH).[1] This enzyme, initially found by Doolittle & Armentrout (1968) in a strain of *Pseudomonas fluorescens* and later purified from other bacteria (Szewczuk & Mulczyk, 1969), was found to be distributed in animal tissues (Szewczuk & Kwiatkowska, 1970). It has recently been purified from guinea pig brain (Browne & O'Cuinn, 1983). It might differ from a M_r 260 000 TRH-degrading serum enzyme which also cleaves the pyroglutamyl residue from TRH (Bauer & Kleinkauf, 1980; Taylor & Dixon, 1978). Pyroglutamyl-peptide hydrolase can catalyze the removal of the N-terminal pyroglutamyl residue from proteins such as fibrinogen and human serum mucoid and also from fibrinopeptides (Szewczuk & Mulczyk, 1969; Ar-

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[1] Abbreviations: TRH, thyrotropin-releasing hormone; LH-RH, luteinizing hormone-releasing hormone; DNPH, 2,4-dinitrophenylhydrazine; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; TCA, trichloroacetic acid; PTFA, pyridinium trifluoroacetate; HMe_3Si, trimethylsilane; TLC, thin-layer chromatography; <Glu, pyroglutamate; 2NA, 2-naphthylamide; pNA, *p*-nitroanilide; SM, sulfamethoxazole; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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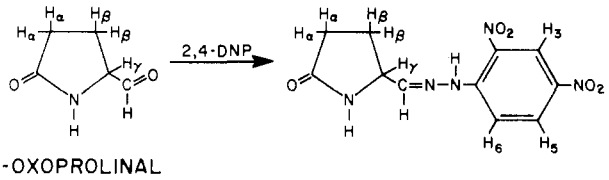

FIGURE 1: Structures of 5-oxoprolinal and the 2,4-dinitrophenylhydrazone derivative of 5-oxoprolinal. Hydrogens are labeled for NMR data (see Materials and Methods).

mentrout & Doolittle, 1969; Orlowski & Meister, 1971). Because of this, initial interest in the enzyme centered on its use as a tool for the removal of the N-terminal pyroglutamyl residue from proteins and peptides in the process of determination of their amino acid sequences.

The physiological role of pyroglutamyl-peptide hydrolase is not known. With the finding, however, that many biologically active peptides including TRH, LH-RH, neurotensin, and bombesin contain N-terminal pyroglutamyl residues, the potential role of the enzyme in the degradation and termination of action of these peptides has attracted interest. A specific and potent inhibitor of pyroglutamyl-peptide hydrolase would be a helpful probe in studies investigating the physiological role of this enzyme. Irreversible inhibitors of the bacterial enzyme have been synthesized (Fujiwara et al., 1981a,b, 1982). The L-pyroglutamyl chloromethyl ketone inhibitor (Fujiwara et al., 1981a) was found to be an effective inhibitor of the rat liver and kidney enzymes; however, the in vivo effects of these inhibitors have not been ascertained. Peptide aldehyde analogues of good substrates are potent and specific inhibitors of thiol and serine proteases (Westerik & Wolfenden, 1972; Thompson, 1973). We have previously synthesized Z-Pro-prolinal as a specific active site directed inhibitor of prolyl endopeptidase (EC 3.4.21.26) (Wilk & Orlowski, 1983). Z-Pro-prolinal was found to be a potent inhibitor both in vitro ($K_i \simeq 14$ nM) (Wilk & Orlowski, 1983) and in vivo (Friedman et al., 1984a).

Since currently available evidence suggests that pyroglutamyl-peptide hydrolase is a thiol protease, we considered the possibility that 5-oxoprolinal (Figure 1), the aldehyde analogue of 5-oxoproline,[2] would be a transition-state inhibitor of pyroglutamyl-peptide hydrolase. Here we report the synthesis of 5-oxoprolinal and show that this compound is a potent inhibitor of pyroglutamyl-peptide hydrolase in both in vitro and in vivo experiments.

Materials and Methods

L-Pyroglutamate, dimethyl sulfoxide, dithiothreitol, Leu-pNA, and LH-RH were obtained from Sigma Chemical Co. (St. Louis, MO). Thionyl chloride, trifluoroacetate, and pyridine were obtained from Fisher Scientific Corp. (Fairlawn, NJ). Sodium borohydride, *N,N'*-dicyclohexylcarbodiimide, *N*-methylpyrrole-2-carboxaldehyde, and 2-thiophenecarboxaldehyde were obtained from Aldrich Chemical Co. (Milwaukee, WI). <Glu-2NA was obtained from United States Biochemical Corp. (Cleveland, OH). Gly-Pro-2NA was obtained from Bachem Chemicals (Torrance, CA). Z-Gly-Pro-SM (Orlowski et al., 1979) and Z-Pro-prolinal (Wilk & Orlowski, 1983) were synthesized as described previously. Silica gel (40-μm average particle diameter) was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Silica-coated plates (Polygram Sil G/UV 254, 40 mm × 80 mm) for

[2] Synonyms of 5-oxoproline are pyrrolidonecarboxylic acid, pyroglutamic acid, and pyroglutamate. Synonyms of pyroglutamyl-peptide hydrolase are pyrrolidonyl peptidase and pyroglutamyl aminopeptidase.

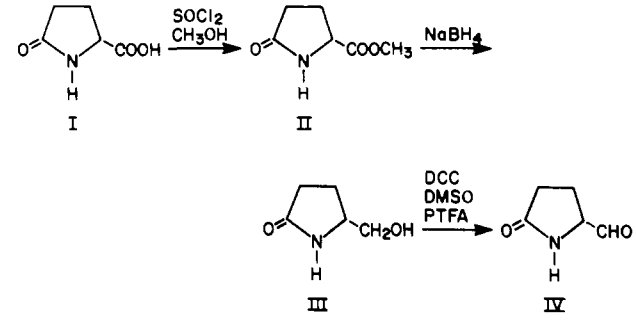

FIGURE 2: Synthesis scheme for 5-oxoprolinal.

thin-layer chromatography were obtained from Brinkmann Instruments (Westbury, NY). Dimethyl sulfoxide was redistilled over NaOH and stored under nitrogen in a flask containing dried 4-Å molecular sieves. Pyridine was redistilled over KOH and stored under nitrogen in a flask containing dry KOH.

NMR spectra were performed in $CDCl_3$ on a Varian FT 80-A instrument and are reported in ppm downfield from $HMe_3Si = 0$. IR spectra were performed on a Beckman IR8 instrument; only diagnostic peaks are reported.

Synthesis of 5-Oxoprolinal. 5-Oxoprolinol (Figure 2, structure III) [5-(hydroxymethyl)pyrrolidinone] was synthesized from 5-oxoproline (I) (pyroglutamate) according to the method of Saijo et al. (1980). Thionyl chloride (8.9 g, 75 mmol) was added dropwise to a stirring solution of L-pyroglutamate (I) (12.9 g, 100 mmol) dissolved in 120 mL of methanol and maintained at −20 °C. After 30 min, the solution was brought to room temperature and stirred for 3 h. After removal of the solvent, the resulting oil was vacuum distilled (145–150 °C, 4 mmHg), yielding 10.34 g (72.3 mmol) of the methyl ester of pyroglutamate (II).

The methyl ester of pyroglutamate (II) (7.4 g, 51.7 mmol) was dissolved in 72 mL of ethanol. The stirred solution was placed in an ice bath, and sodium borohydride (1.96 g, 51.7 mmol) was added over a period of 30 min. The reaction was continued for 2 more hours at room temperature. The mixture was then acidified with concentrated HCl and filtered, and the filtrate was evaporated under reduced pressure to give an oil which was chromatographed on a silica gel column (2 × 25 cm). The column was eluted with ethyl acetate followed by elution with a mixture of ethyl acetate–methanol (90:10). An oil (III) was obtained which was crystallized from an ethyl acetate–chloroform mixture. A white solid with a melting point of 72–74 °C was obtained, in agreement with the value reported in the literature (Saijo et al., 1980). Thin-layer chromatography in an ether–2-propanol solvent system (85:15) revealed a single spot (R_f 0.06) when visualized by the chlorine–tolidine method for nitrogen-containing compounds (Krebs et al., 1969).

The alcohol (III) was oxidized to the aldehyde derivative, 5-oxoprolinal (IV), by using a modification of the methods of Pfitzner & Moffat (1965) and Jones & Wigfield (1966). A weak acid, pyridinium trifluoroacetate (PTFA), prepared according to Bourne et al. (1954), was the catalyst. *N,N'*-Dicyclohexylcarbodiimide (2.7 g, 13 mmol) was added to redistilled Me_2SO (12.5 mL, 175 mmol). After the solution was stirred briefly, 5-oxoprolinol (III) (230 mg, 2 mmol) followed by PTFA (240 mg, 1.25 mmol) was added. The pH remained close to neutral, a condition that is necessary because the pyrrolidone ring is labile to acidic and basic conditions. The aldehyde was quantitated by the formation of the 2,4-dinitrophenylhydrazone derivative with butyraldehyde as standard (Reingold & Orlowski, 1979). After 6 h, no further

increase in aldehyde could be detected. The highest yield obtained from this reaction was about 35%.

Chloroform was added, and the reaction mixture was filtered. The filtrate was evaporated under reduced pressure at room temperature to remove the chloroform and at 55 °C under vacuum for a short period of time to remove some of the Me_2SO. The oil was chromatographed on a silica gel column (2.5 × 85 cm) and eluted stepwise with ether–2-propanol (0–15% 2-propanol). The eluate was monitored for aldehyde by a 2,4-dinitrophenylhydrazine (DNPH) spray (0.4% in 2 N HCl). The aldehyde emerged with ether–2-propanol (85:15). Removal of the solvent of the pooled aldehyde-containing fractions yielded an oil (IV). TLC analysis in an ether–2-propanol system (85:15) revealed one DNPH-positive spot with an R_f value of 0.20. When visualized with the tolidine spray, however, a fast-moving spot was also present. To remove this contaminant, the oil was purified by using a flash chromatography (Still et al., 1978) silica gel column (1.5 × 60 cm) equilibrated with a mixture of chloroform–ethanol (95:5) and eluted under nitrogen pressure with this solvent. The resulting oil, when chromatographed on a TLC plate, afforded only one spot when visualized with the tolidine spray, which coincided with the spot for aldehyde visualized by the DNPH spray. This indicated that no other nitrogen-containing compound was present. The amount of aldehyde, however, as quantitated by 2,4-dinitrophenylhydrazone formation with butyraldehyde as a standard was less than the weight of the oil, possibly due to incomplete removal of solvent(s): ^{1}H NMR ($CDCl_3$) δ 2.38 (m, $H_{\alpha'\alpha}/H_{\beta'\beta}$), 4.22 (m, H_γ), 9.61 (br s, CHO); IR ν_{max} (oil, NaCl salt plates) 3400 (w, NH), 1680 cm^{-1} (m, CONH overlap CHO).

Synthesis of the 2,4-Dinitrophenylhydrazone Derivative of 5-Oxoprolinal. Partially purified 5-oxoprolinal (IV) (75 mg, 0.66 mmol), obtained from the first silica gel chromatography step, was dissolved in 4 mL of 95% ethanol. A DNPH solution was prepared by dissolving DNPH (0.4 g, 2 mmol) in 2 mL of concentrated H_2SO_4 and adding 3 mL of water and 10 mL of 95% ethanol. Three milliliters of this solution was added to the aldehyde solution. A solid precipitated immediately which was filtered, dissolved in chloroform, and chromatographed on a silica gel column. The column was washed with chloroform, and the DNPH derivative was then eluted with a mixture of chloroform–ethanol (95:5). The pure DNPH derivative, as determined by TLC (R_f value of 0.11 by using a chloroform–ethanol mixture, 95:5), eluted as the second component from the column. Removal of the solvent yielded a yellow solid with a melting point of 175–177 °C. The NMR of the solid confirmed that the 2,4-dinitrophenylhydrazone derivative of 5-oxoprolinal was formed (Figure 1): ^{1}H NMR (in $CDCl_3$) δ 2.50 (br t, J = 5.6 Hz, $H_{\alpha'\alpha}/H_{\beta'\beta}$, 4.55 (m, H_γ), 5.83 (br s, exchange with D_2O, lactam NH), 7.40 (d, J = 4.9 Hz, CH=N), 7.90 (d, J = 9.5 Hz, H_6), 8.40 (dd, J_o = 9.5 Hz, J_m = 3.0 Hz, H_5), 9.10 (d, J = 3.0 Hz, H_3), 11.10 (br s, exchange with D_2O, N–NH); IR ν_{max} ($CHCl_3$ solution, NaCl cell with a 0.2-mm path length) 3480, 3460 (w, NH), 3350 (w, NH), 1690 (m, CONH), 1650, 1630 (s, C=N), 1550 cm^{-1} (s, NO_2). Anal. Calcd for $C_{11}N_5O_5H_{11}$: H, 3.78; C, 45.05; N, 23.88. Found: H, 3.82; C, 45.18; N, 23.81.

A standard curve prepared by reacting known amounts of the solid 2,4-dinitrophenylhydrazone derivative of 5-oxoprolinal with the Cellosolve reagent (Reingold & Orlowski, 1979) was found to be similar to the curve with butyraldehyde as standard.

Enzyme Preparations. Calf liver pyroglutamyl-peptide hydrolase (pyroglutamate aminopeptidase) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Prolyl endopeptidase was purified to apparent homogeneity as described by Wilk & Orlowski (1983). Aminopeptidase M (EC 3.4.11.2) was purified from hog kidney by a modification of the method of Pfleiderer (1970) as described (Friedman et al., 1984b). Diaminopeptidase IV (EC 3.4.14.1) was purified to apparent homogeneity from rabbit kidney cortex essentially as described by Yoshimoto & Walter (1977).

Determination of Enzymatic Activities. The activities of all enzymes were measured spectrophotometrically by determining the release of aromatic amines from the appropriate chromogenic substrate, according to the method of Bratton & Marshall (1939) as modified by Goldbarg & Rutenburg (1958). One unit of activity is defined as the amount of enzyme needed to release 1 μmol of chromogen/h at 37 °C under the conditions of the assay. All assays were run in triplicate.

Enzymatic Assays. (a) Pyroglutamyl-peptide hydrolase was assayed with the substrate <Glu-2NA. The incubation mixture (final volume 250 μL) contained 10 μL of 10 mM <Glu-2NA (in Me_2SO), 20 μL of 20 mM DTT, 20 μL of 20 mM EDTA (pH 7.2), 50 mM Tris-HCl buffer (pH 7.5), and 50 μL of lyophilized calf liver enzyme reconstituted in the above buffer (3.6 mg of enzyme/mL) immediately before assaying. Incubations were carried out for 1 h at 37 °C and were stopped by adding 250 μL of 10% TCA.

(b) Prolyl endopeptidase activity was determined with the substrate Z-Gly-Pro-SM, as described by Orlowski et al. (1979). The incubation mixture (final volume 250 μL) contained 10 μL of 10 mM DTT, 50 μL of purified prolyl endopeptidase (0.15 unit), 0.1 M Tris-HCl buffer (pH 8.3), and 50 μL of Z-Gly-Pro-SM (2.7 mg/mL in 0.1 M Tris-HCl, pH 8.3). Incubations were for 15 min at 37 °C. The reaction was stopped by addition of 250 μL of 10% TCA, and the release of free SM was quantitated by diazotization.

(c) Aminopeptidase M activity was determined with the substrate Leu-pNA. The incubation mixture (final volume 250 μL) contained 0.05 M Tris-HCl buffer (pH 7.5), 10 μL of purified aminopeptidase M (0.14 unit), and 10 of μL of 10 mM Leu-pNA (in methanol). Incubations were for 10 min at 37 °C. The reaction was stopped by the addition of 250 μL of 10% TCA, and the release of free pNA was quantitated by diazotization.

(d) Diaminopeptidase IV activity was determined with the substrate Gly-Pro-2NA. The incubation mixture (final volume 250 μL) contained 0.05 M Tris-HCl buffer (pH 7.5), 10 μL of purified diaminopeptidase IV (0.084 unit), and 10 μL of 10 mM Gly-Pro-2NA (in Me_2SO). Incubations were for 30 min at 37 °C. The reaction was stopped by the addition of 250 μL of 10% TCA, and the release of free 2NA was quantitated by diazotization.

Kinetic Studies of Inhibitors. All enzymes were preincubated with inhibitor at 37 °C for 10 min. The reactions were initiated by addition of substrate. The K_i of 5-oxoprolinal for pyroglutamyl-peptide hydrolase was calculated by the methods of Dixon (1953) and Henderson (1972). The K_i of other inhibitors was calculated by the method of Dixon (1953).

LH-RH Degradation. LH-RH degradation was studied with purified calf liver pyroglutamyl-peptide hydrolase. Z-Pro-prolinal was added to prevent degradation by any contaminating prolyl endopeptidase. Incubation mixtures contained 31 μL of LH-RH (62.5 μg) 10 μL of Z-Pro-prolinal (final concentration 10–5 M), 20 μL of 20 mM DTT, 20 μL of 20 mM EDTA (pH 7.2), Tris-HCl buffer (pH 7.5), and 50 μL of pyroglutamyl-peptide hydrolase (3.6 mg of lyophilized

powder/mL of buffer). Incubation tubes contained either 25 μL of 5-oxoprolinal (final concentration 10–6 M) or the equivalent amount of buffer. Incubations were carried out at 37 °C, and the reaction was monitored by using a Perkin-Elmer series 2 HPLC equipped with a variable-wavelength spectrophotometric detector (LC-55). A total of 20 μL of incubation mixture was chromatographed on a 250 × 4 mm Bio-Sil OD-5S reverse-phase column (Bio-Rad Laboratories, Richmond, CA) as described by Wilk & Orlowski (1982). After 3 h of incubation, most of the LH-RH was converted to product in the tube without inhibitor. The incubation mixture in the sample lacking inhibitor was then applied to the column, and the product peak (retention time of 9 min) was collected, hydrolyzed, and analyzed on a Technion TSM amino acid autoanalyzer as described by Wilk & Orlowski (1982).

Animal Experiments. Male Swiss Albino mice weighing approximately 35 g were used for all experiments. They were fed a commercial Purina laboratory chow diet. 5-Oxoprolinal (50 mg/kg in 50% ethanol) or vehicle was administered by intraperitoneal injection. After 10 or 30 min, mice were killed by cervical dislocation. The tissues were immediately removed and placed on ice. After addition of 5 volumes of ice-cold 0.05 M Tris-HCl buffer (pH 7.5), the tissues were disrupted by using a Polytron homogenizer (Brinkmann Instruments) and then homogenized in an ice-cooled homogenizer equipped with a Teflon pestle. Pyroglutamyl-peptide hydrolase activity in the homogenates was determined as described above with 50 μL of homogenate. After a 1-h incubation at 37 °C, the reaction was stopped by addition of 250 μL of 10% TCA. Control tubes in which the enzyme and substrate were omitted separately were also carried through the procedure. Tubes were then centrifuged, and 375 μL of of supernatant of all organs except liver and kidney was removed. For liver and kidney incubations, 75 μL of supernatant was diluted with 300 μL of 0.05 M Tris-HCl buffer (pH 7.5). 2NA concentrations were determined by using a procedure similar to that described by Friedman et al. (1984a) for the measurement of sulfamethoxazole. The chromogen was measured at 580 nm. Specific activities were expressed in terms of nanomoles of 2NA per gram of tissue per hour. The activity of pyroglutamyl-peptide hydrolase in animals receiving 5-oxoprolinal was compared to the activity of control animals by using the one-tailed Student's *t* test.

Results

5-Oxoprolinal was found to be a potent inhibitor of pyroglutamyl-peptide hydrolase in vitro. Analysis by the method of Dixon (1953) revealed a K_i of 20 nM (mean of three experiments) (see Figure 3) with the intersection of the lines of different substrate concentrations occurring above the *x* axis, a condition indicative of competitive inhibition. The analysis of the kinetics of this tight binding inhibitor was also carried out by the method of Henderson (1972). In this method, the inhibitor concentration divided by the degree of inhibition is plotted on the ordinate, and the velocity without inhibitor divided by the velocity with inhibitor is plotted on the abscissa. A linear plot was obtained for three different substrate concentrations, and the slope was found to increase with increasing substrate concentrations, a condition indicative of competitive inhibition. When the slope of each of the three lines was plotted vs. the substrate concentration, a linear plot was obtained with the *y* intercept of 26 nM equal to the K_i.

Other compounds related to 5-oxoprolinal were tested for their ability to inhibit pyroglutamyl-peptide hydrolase activity. As shown in Table I, pyroglutamic acid (5-oxoproline) and

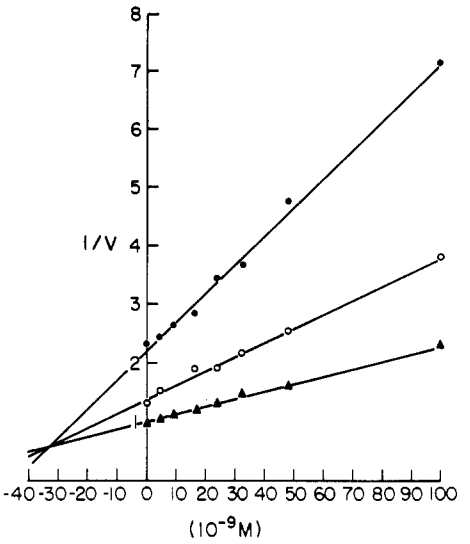

FIGURE 3: Dixon plot of the inhibition of pyroglutamyl-peptide hydrolase by 5-oxoprolinal. The inhibitor was preincubated for 10 min at 37 °C with the enzyme, and the reaction was initiated by addition of <Glu-2NA as described under Materials and Methods. (▲) Concentration of substrate = 0.4 mM; (○) concentration of substrate = 0.2 mM; (●) concentration of substrate = 0.1 mM.

Table I: Effect of Inhibitors on Pyroglutamyl-Peptide Hydrolase Activity[a]

inhibitor	K_i (mM)
Z-Pro-prolinal	7.5
5-oxoproline	0.85
5-oxoprolinol	0.65
5-oxoprolinal	2.6×10^{-5}
2-thiophenecarboxaldehyde	NI[b]
N-methylpyrrole-2-carboxaldehyde	NI[b]

[a] The K_i of pyroglutamyl-peptide hydrolase was determined as described under Materials and Methods. The enzyme was preincubated with inhibitor for 10 min prior to addition of substrate. K_i values were determined by the method of Dixon (1953). [b] NI, did not inhibit at a final concentration of 0.4 mM.

the alcohol derivative of pyroglutamic acid (5-oxoprolinol) inhibited enzymatic activity with a K_i of 0.85 mM and 0.65 mM, respectively. These K_i's are over 10000-fold higher than that of the aldehyde. The K_i of Z-Pro-prolinal, the specific aldehyde inhibitor of prolyl endopeptidase (Wilk & Orlowski, 1983), for pyroglutamyl-peptide hydrolase was found to be 7.5 mM. This is over 5 orders of magnitude higher than its K_i for prolyl endopeptidase. Two other five-membered heterocyclic compounds with an aldehyde carbonyl in the 2-position were also tested for their effect on pyroglutamyl peptide hydrolase activity. As shown in Table I, 2-thiophenecarboxaldehyde and *N*-methylpyrrole-2-carboxaldehyde at concentrations of 0.4 mM did not inhibit pyroglutamyl-peptide hydrolase activity.

5-Oxoprolinal was tested on other peptidases that might be considered as susceptible to inhibition by this aldehyde. The K_i of 5-oxoprolinal on prolyl endopeptidase was found to be 0.36 mM, which is over 4 orders of magnitude greater than its K_i for pyroglutamyl-peptide hydrolase. At a concentration of 10–5 M, 5-oxoprolinal did not inhibit aminopeptidase M or diaminopeptidase IV (post-proline diaminopeptidase) activities.

Since 5-oxoprolinal was found to inhibit the cleavage of <Glu-2NA by pyroglutamyl-peptide hydrolase, we chose to study the effect of the inhibitor on the cleavage of a biological peptide by this enzyme. LH-RH is cleaved by pyroglutamyl-peptide hydrolase between the pyroglutamyl and

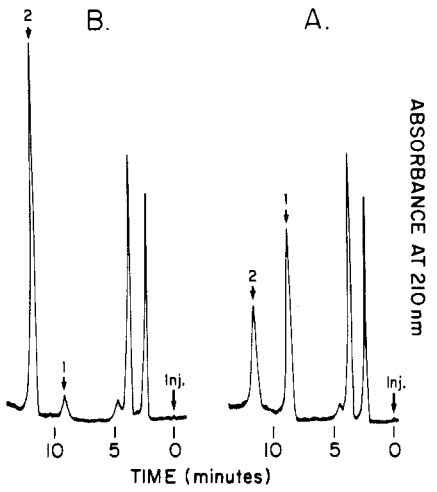

FIGURE 4: HPLC chromatogram of LH-RH incubated with pyroglutamyl-peptide hydrolase for 3 h at 37 °C as described under Materials and Methods. (A) Incubation without 5-oxoprolinal. (B) Incubation in the presence of 5-oxoprolinal (10^{-6} M). A 20-μL aliquot of each incubation mixture was chromatographed on a 250 × 4 mm Bio-Sil OD-5S reverse-phase column equilibrated with a mixture of acetonitrile and potassium phosphate buffer (0.05 M, pH 2.0). The starting concentration of acetonitrile was 20% and was linearly increased at a rate of 1%/min. The flow rate was 1 mL/min. Absorbance was monitored at 210 nm. Peak 1 is des-<Glu^1-LH-RH, and peak 2 is LH-RH.

histidine residues (Bauer et al., 1981). HPLC analysis of an incubation mixture containing LH-RH and pyroglutamyl-peptide hydrolase revealed a peak histidyl to intact LH-RH (retention time = 12 min). This peak decreased with time concomitantly with the appearance of a new peak with a retention time of 9 min which progressively increased with time. As shown in Figure 4A, after 3 h there was only about 25% of intact LH-RH remaining. The peak that appeared during the incubation was collected, hydrolyzed, and analyzed on an amino acid analyzer. Analysis of the amino acid composition of the product was consistent with the structure of des-<-Glu^1-LH-RH, confirming that cleavage between the 1-position and 2-position occurred. As shown in Figure 4B, 5-oxoprolinal at a concentration of 10^{-6} M almost totally blocked the degradation of LH-RH by pyroglutamyl-peptide hydrolase. After 3 h, mainly intact LH-RH was present.

5-Oxoprolinal was injected into mice to determine if the inhibitor was effective in vivo. The results shown in Table II demonstrate greater than 60% inhibition of enzymatic activity in all organs tested 10 min after 5-oxoprolinal injection compared with control activity. This inhibition was statistically significant in all organs tested. After 30 min, the degree of inhibition decreased, but significant inhibition occurred in all organs except the heart. The highest degree of inhibition at both times was in the kidney.

Table II: Pyroglutamyl-Peptide Hydrolase Activity in Mouse Tissues after Intraperitoneal Administration of 5-Oxoprolinal[a]

	enzymatic activity (nmol of 2NA g^{-1} h^{-1})		
		time after inhibitor administration	
tissue	control	10 min	30 min
brain	540 ± 100 (4)	210 ± 24[c] (7)	300 ± 70[b] (4)
heart	870 ± 70 (4)	220 ± 55[d] (7)	720 ± 160 (4)
muscle	560 ± 110 (4)	75 ± 190[d] (7)	220 ± 50[b] (4)
lung	980 ± 130 (4)	120 ± 27[d] (7)	450 ± 50[b] (4)
spleen	1100 ± 230 (4)	64 ± 11[d] (7)	540 ± 120[b] (4)
liver	4600 ± 980 (4)	1600 ± 340[b] (4)	2200 ± 85[b] (4)
kidney	8800 ± 2500 (4)	250 ± 69[c] (4)	565 ± 70[b] (3)

[a] 5-Oxoprolinal (50 mg/kg) was administered intraperitoneally as a solution in 50% ethanol. Control animals received the vehicle; 10 and 30 min after injection, the animal was sacrificed, and enzymatic activities were determined as described under Materials and Methods. Data are mean values ±SE. Values in parentheses represent the number of animals used. The level of significance was found by comparing the activities of inhibitor-treated homogenates with that of control homogenates with the use of the one-tailed Student's t test. [b] $P < 0.05$, significantly different from controls. [c] $P < 0.01$. [d] $P < 0.001$.

DISCUSSION

Peptide aldehyde analogues of the acyl portion of protease substrates are potent competitive inhibitors of serine and thiol proteases (Westerik & Wolfenden, 1972; Thompson, 1973). Since pyroglutamyl-peptide hydrolase specifically hydrolyzes pyroglutamate from the N-terminus of peptides by a mechanism in which a cysteine residue in the active site is believed to be necessary for activity, it was expected that the aldehyde analogue of pyroglutamate should form a tetrahedral thiohemiacetal with the cysteine in the active site. The observation that the K_i of 5-oxoprolinal is about 4 orders of magnitude lower than that of the corresponding alcohol or acid is consistent with the interpretation that an analogue of the transition-state intermediate is being formed in the interaction of the inhibitor with the enzyme.

5-Oxoprolinal was found to act as a competitive inhibitor as demonstrated by the Dixon and Henderson plots (Figure 2). This is in contrast to Z-Pro-prolinal which was found to be a noncompetitive inhibitor of prolyl endopeptidase (Wilk & Orlowski, 1983). Although the K_i for 5-oxoprolinal (26 nM) is similar to that of Z-Pro-prolinal (14 nM), 5-oxoprolinal appears to be a less potent and shorter acting inhibitor in vivo. The degree of inhibition was much lower at 30 min than at 10 min for 5-oxoprolinal while the degree of in vivo inhibition for Z-Pro-prolinal remained high at 30 min (Friedman et al., 1984a). At a dose of 5 mg/kg, Z-Pro-prolinal inhibited greater than 90% of prolyl endopeptidase activity in most organs after 30 min, while a dose of 50 mg/kg of 5-oxoprolinal gave between 17% and 94% inhibition depending on the organ after 30 min. The lower potency and shorter duration of 5-oxoprolinal inhibition might be due to the fact that its binding to the enzyme is readily reversible and also that its metabolic inactivation and elimination might proceed faster than that of Z-Pro-prolinal. It may be expected that alcohol dehydrogenase and aldehyde oxidase might contribute to the metabolism of the inhibitor.

It is possible that the inhibition of the enzyme in homogenates after intraperitoneal administration of the inhibitor does not reflect true in vivo inhibition but rather results from exposure of the enzyme to the inhibitor only after disruption of the tissue by homogenization. This possibility is especially relevant since the dose used was relatively high. To exclude this possibility, future studies in which the inhibitor is used to block the degradation of a substrate of pyroglutamyl-peptide hydrolase in vivo need to be carried out.

The synthesis of 5-oxoprolinal proved to be quite difficult. Although the Me_2SO–DCC method gave low yields and impurities which comigrated with 5-oxoprolinal on silica gel columns, it was more successful than other methods including reduction of pyroglutamate by the thexylborane reagent (Brown et al., 1972), reduction of the ester derivative by $LiAlH_4$ (Brown et al., 1982), and oxidation of the alcohol derivative by pyridinium dichromate (Stanfield et al., 1981) or by alcohol dehydrogenase (Andersson & Wolfenden, 1982). 5-Oxoprolinal appears to be labile to acidic or basic conditions, possibly due to lactam ring opening. The use of the neutral catalyst PTFA increased the yield of the Me_2SO–DCC oxidation. Attempts to catalyze the Me_2SO–DCC oxidation by

oxalic acid (Omura & Swern, 1978), dichloroacetic acid (Thompson, 1973), and phosphoric acid (Thompson, 1977) met wtih little success.

The NMR and IR spectra are consistent with the structure of 5-oxoprolinal as depicted in Figure 1. To confirm this structure, the 2,4-dinitrophenylhydrazone derivative of this compound was prepared and isolated as a solid. The NMR and IR spectra and the elemental analysis are correct for the derivative's structure depicted in Figure 1. The derivative was used to prepare a standard curve for the 5-oxoprolinal assay and used to quantitate 5-oxoprolinal in subsequent preparations. The curve was found to be similar to that of butyraldehyde, which confirms that the DNPH reaction using butyraldehyde accurately quantitated 5-oxoprolinal in the earlier experiments. Since α-aminoaldehydes are particularly prone to racemization and since this tendency is fostered by exposure to silica gel (Hamada & Shiori, 1982), it is likely that 5-oxoprolinal is a racemate.

The enzyme studied in this paper has often been referred in the literature as pyroglutamyl aminopeptidase. This term implies that the enzyme cleaves free N-terminal amino acids from peptides. Since it only cleaves a blocked N-terminal amino acid, we have preferred to refer to this enzyme as pyroglutamyl-peptide hydrolase as suggested by Kwiatkowska et al. (1974).

In the literature, there are reports of a membrane-bound pyroglutamyl-peptide hydrolase detected in guinea pig brain (Browne et al., 1981; Hayes et al., 1979) that may be similar to the TRH-degrading serum enzyme (Bauer & Kleinkauf, 1980; Taylor & Dixon, 1978). If these enzymes are indeed metalloenzymes, they should be resistant to inhibition by 5-oxoprolinal. 5-Oxoprolinal would be a valuable tool for the study of the properties of these serum and membrane-bound brain enzymes.

In tissues, two major enzymes appear to be responsible for the initial degradation of TRH: deamidation catalyzed by prolyl endopeptidase and removal of the N-terminal pyroglutamyl residue catalyzed by pyroglutamyl-peptide hydrolase (Griffiths & Kelley, 1979; Kreider et al., 1981; Busby et al., 1982; Bauer & Kleinkauf, 1980). With the synthesis of 5-oxoprolinal and Z-Pro-prolinal, potent and specific inhibitors of the two enzymes are now available. These aldehydes inhibit brain prolyl endopeptidase and pyroglutamyl-peptide hydrolase, respectively in vivo. Their selective use should therefore determine the relative importance of these two enzymes in the in vivo degradation of TRH. Z-Pro-prolinal and 5-oxoprolinal might be useful in studies on TRH turnover or in studies on the biosynthesis of TRH in which it would be desirable to prevent TRH degradation. 5-Oxoprolinal could also be used to evaluate the role of pyroglutamyl-peptide hydrolase in the degradation of other pyroglutamyl-containing peptides such as neurotensin, LH-RH, and bombesin. The physiological and behavioral consequences of 5-oxoprolinal administration are also worthy of investigation.

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Electron-Transfer Flavoprotein-Ubiquinone Oxidoreductase from Pig Liver: Purification and Molecular, Redox, and Catalytic Properties†

Joe D. Beckmann‡ and Frank E. Frerman*
Department of Microbiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226
Received December 10, 1984

ABSTRACT: Electron-transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) was purified to homogeneity from pig liver submitochondrial particles. It is comparable in molecular weight and general properties to ETF-QO from beef heart [Ruzicka, F. J., & Beinert, H. (1977) *J. Biol. Chem. 252*, 8440–8445], and the electron spin resonance signals of the reduced iron–sulfur cluster are essentially identical. ETF-QO catalyzes the transfer of electrons from electron-transfer flavoprotein (ETF) to nitro blue tetrazolium, with a sluggish reaction turnover number of about 10–30 min^{-1}. In contrast, the enzyme rapidly disproportionates ETF semiquinone, with a turnover number of 200 s^{-1}. The reverse reaction, comproportionation of oxidized and hydroquinone ETF, provides an enzymatic assay for ETF-QO with picomolar sensitivity. Equilibrium spectrophotometric titrations show that ETF-QO accepts a maximum of two electrons from ETF and accepts three electron equivalents from dithionite or by photochemical reduction. All electrons from the enzymatically or chemically reduced protein can be transferred to 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone (PB), and this reaction is readily reversible. Reduction of ETF-QO by 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzohydroquinone is pH dependent and indicates the enzyme to have a redox potential that decreases by 47 mV per pH unit. Therefore, ETF-QO binds one to two protons upon reduction. The E_0' at pH 7.3 is 38 mV. The ability of ETF-QO to catalyze the equilibration of ETF redox states has been used to evaluate the equilibrium $2ETF_{sq} + nH^+ \rightleftarrows ETF_{ox} + ETF_{hq}$. The pH dependence of the equilibrium indicates that $n = 1$ and is consistent with the assignment of ETF semiquinone (ETF_{sq}) and hydroquinone flavin species as true anions. The one-electron reduction potential of oxidized ETF is predicted to be independent of pH.

As many as eight primary flavoprotein dehydrogenases of the mitochondrial matrix (Crane et al., 1956; Hauge et al., 1956; Noda et al., 1980; Ikeda et al., 1983; Ikeda & Tanaka, 1983; Frisell & MacKenzie, 1962) are oxidized by a common flavoprotein, electron-transfer flavoprotein (ETF).[1] Anaerobic steady-state reduction of ETF by catalytic concentrations of G-AD results in the formation of the one-electron-reduced ETF anionic flavin semiquinone, which is a potent product inhibitor of the primary dehydrogenase (Beckmann et al., 1981). The fully reduced ETF hydroquinone is formed enzymatically but at a 10-fold slower rate subsequent to the one-electron reduction (Reinsch et al., 1980; Hall & Lambeth, 1980). ETF semiquinone is also readily obtained by chemical reduction with dithionite (Gorelick et al., 1982). The stabilization of the radical has been suggested to be kinetic rather than thermodynamic (Massey & Hemmerich, 1980; Hussin et al., 1984), since the semiquinone disproportionates to a mixture of all three redox states over the period of days (Gorelick et al., 1982). Since the equilibrium constant for disproportionation is near 1, it is difficult to evaluate the importance of the thermodynamic properties of ETF redox states in determining how this flavoprotein functions.

After reduction by a primary dehydrogenase, ETF is apparently reoxidized by a 4Fe-4S flavoprotein, ETF-QO (Ruzicka & Beinert, 1975, 1977; Schmidt et al., 1983). The specific function of the iron–sulfur flavoprotein is suggested by three lines of evidence. First, freeze–quench studies with the solubilized ETF-QO demonstrated the kinetic competence of the electron transfer from ETF to ETF-QO (Ruzicka & Beinert, 1977). Second, there is a specific increase of ETF-QO in brown adipose tissue during cold acclimation of guinea pigs. Brown adipose tissue oxidizes fatty acids to support thermogenesis (Flatmark et al., 1982). Third, immunoreactive enzyme is undetectable in liver submitochondrial particles derived from a patient with the heritable metabolic disorder glutaric acidemia type II (Goodman & Frerman, 1984). Patients with

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‡ Present address: Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755.

[1] Abbreviations: ETF, electron-transfer flavoprotein; G-AD, general (or medium-chain) acyl-CoA dehydrogenase; ETF-QO, electron transfer flavoprotein-ubiquinone oxidoreductase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Q, ubiquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; PB, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone; PB-H_2, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzohydroquinone; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; NBT, nitro blue tetrazolium; TN, turnover number; AMPD, 2-amino-2-methyl-1,3-propanediol; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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