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SYNTHESIS AND CHARACTERIZATION OF THE OXYGEN AND DESTHIO ANALOGUES OF GLUTATHIONE AS DEAD-END INHIBITORS OF GLUTATHIONE S-TRANSFERASE

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The oxygen analogue, γ-L-Glu-L-SerGly (GOH)[1] and desthio analogue, γ-L-Glu-L-AlaGly (GH) have been synthesized by a simple three step procedure involving active ester coupling of N-t-BOC-α-(4-nitrophenyl)-L-glutamate to L-SerGly and L-AlaGly, respectively. The two peptides are excellent dead-end inhibitors of isozymes 3-3 and 4-4 of rat liver glutathione S-transferase. At low fixed concentrations of 1-chloro-2,4-dinitrobenzene (CDNB) GOH and GH are linear competitive inhibitors of isozyme 3-3 vs glutathione with K_I values of 13.0 and 116 μM, respectively. Both peptides are non-competitive (mixed-type) inhibitors vs CDNB when glutathione is the fixed substrate. Similar results are obtained with both peptides and isozyme 4-4. The results rule out ordered or ping-pong kinetic mechanisms where the electrophile adds first.

The glutathione S-transferases (EC 2.5.1.18) catalyze the nucleophilic addition of the thiol of glutathione to a wide variety of compounds with electrophilic substituents. The reaction, which is the first step in mercapturic acid synthesis is of considerable importance in the detoxication of alkylating agents (1-3). The kinetic mechanism of several of the isozymes have been examined (4-9) primarily through initial velocity and product inhibition studies but without the benefit of structural analogues of the tripeptide which can be reasonably expected to be recognized by the glutathione binding site only. In addition, little is known about the

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[1]Abbreviations used are: GOH, γ-L-glutamyl-L-serylglycine; GH, γ-L-glutamyl-L-alanylglycine; GSH, glutathione; SDS, sodium dodecyl sulfate; DCC, dicyclohexylcarbodiimide; TMS, tetramethylsilane; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; CDNB, 1-chloro-2,4-dinitrobenzene; MOPS, 3-(N-morpholino)-propanesulfonic acid; PIPES, piperazine N,N'-bis-(2-ethanesulfonic acid); t-BOC, tert-butyloxycarbonyl.

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structural features of glutathione which are important in enzyme-peptide interactions. For this reason we report here a simple synthesis of two analogues of glutathione, the oxygen analogue γ-L-Glu-L-SerGly (GOH) and the desthio analogue γ-L-Glu-L-AlaGly (GH). Although a synthesis of GH, a natural product known as norophthalmic acid (10) has been previously reported (11), to the best of our knowledge a synthesis of GOH has not appeared in the literature. Preliminary data suggest the two peptide analogues are valuable molecules for studies of the chemical and kinetic mechanisms of glutathione dependent enzymes since they are potent dead-end inhibitors of isozymes 3-3 and 4-4 of rat liver glutathione S-transferase.[2]

EXPERIMENTAL METHODS

Materials. Isozymes 3-3 and 4-4 of rat liver glutathione S-transferase were prepared by a modification (14) of the procedure of Mannervik and Jensson (13) and were homogeneous by the criteria of SDS gel electrophoresis and high performance hydroxylapatite chromatography (14). The dipeptides L-SerGly and L-AlaGly, N-t-BOC-α-benzyl-L-glutamic acid, buffer salts and reduced glutathione were obtained from Sigma Chemical Co. Dicyclohexylcarbodiimide, anhydrous trifluoroacetic acid, 1-chloro-2,4-dinitrobenzene and 4-nitrophenol were from Aldrich Chemical Co. Pyridine was refluxed and distilled from barium oxide before use.

Synthesis of N-t-BOC-α-benzyl-γ-(4-nitrophenyl)-L-glutamate. The protected active γ-ester of L-glutamic acid was prepared by the general method of Bodanszky and du Vigneaud (15). To a stirred solution of 5.06 g (15 mmol) N-t-BOC-L-glutamic acid α-benzyl ester and 2.55 g (18 mmol) 4-nitrophenol in 40 mL ethylacetate at 0 °C was added 3.1 g (15 mmol) DCC. The mixture was stirred for 0.5 h at 0 °C then 1.5 h at room temperature at which point 25 μL glacial acetic acid was added. After stirring for 5 min. the dicyclohexylurea was removed by filtration. The oily residue obtained after evaporation of the solvent from the filtrate was dissolved in 100 mL hot, dry ethanol and allowed to crystallize (needles) overnite at 4°C to give 5.78 g (12.6 mmol, 84%) of the active γ-ester (m.p. 99-100 °C).

Synthesis of N-t-BOC-α-benzyl-γ-L-glutamyl-L-serylglycine. Coupling of the protected active γ-ester to L-serylglycine was accomplished in aqueous pyridine by the method of Bodanszky et al. (16). A stirred solution of 0.5 g L-serylglycine (3.08 mmol) in 6 mL 33% pyridine in H_2O at room temperature was adjusted to pH 8.7 (glass electrode). A total of 1.2 equiv. of active ester (1.64 g, 3.7 mmol) in 4 mL dry pyridine was added in five equal aliquots over a 8 h period and the mixture was stirred an additional 8 h. The pH was maintained at 8.5 during the coupling by periodic addition of 2.5 N NaOH. After the reaction mixture ceased consumming base (~16 h) 3 g $NaHCO_3$ in 25 mL H_2O was added and the mixture was extracted with ethyl acetate. The organic layer was back extracted with 10 mL H_2O. The two aqueous fractions were combined and adjusted to pH 2.5 with 3.0 N HCl and the oily product granulated upon storage at 4 °C to give 1.34 g (2.8 mmol) of the crude protected peptide, 91% yield based on SerGly. Analytically pure samples of the protected

[2]Nomenclature for the glutathione S-transferases is that recently proposed by Jakoby et. al. (12). Isozyme 3-3 is identical with isozyme A_2 (13) and isozyme A (4). Isozyme 4-4 is isozyme C_2 (13).

tripeptide could be obtained by reversed phase HPLC on a 1.0 x 25 cm Altex Ultrasphere ODS column eluted at 2.0 mL/min with 50% CH_3OH, 50% 0.1 M acetic acid for 15 min followed by a gradient of 1%/min to 65% CH_3OH. Retention time of the protected tripeptide was 30 min. Amino acid analysis: Gly 1.00, Glu 1.01, Ser 0.89.

Synthesis of γ-L-glutamyl-L-serylglycine. 500 mg (1.04 mmoles) of the protected peptide under N_2 was dissolved in 25 mL anhydrous trifluoroacetic acid at 0 °C. Anhydrous HBr was bubbled through the solution for 30 min at 0 °C then at room temperature for 2 h. N_2 was bubbled through the solution for 30 min. Trifluoroacetic acid was removed by flash evaporation to give a tan oil which solidified upon addition of diethylether. The precipitate was washed three times with ether and dried in a vacuum dessicator to give 360 mg (0.97 mmoles, 93%) of γ-L-Glu-L-SerGly as the hydrobromide salt. The product was purified by chromtography on a 2.5 x 35 cm bed of Bio Rad AG1-X2 acetate eluted with 200 mL of 50 mM acetic acid followed by a 2 L linear gradient of 50 mM to 1 M acetic acid. Product which eluted between 1,300 and 1,460 mL was lyophilized three times with H_2O to give 257 mg (0.88 mmol, 85%) of a white hygroscopic powder. Amino acid analysis: Gly 1.00, Glu 1.00, Ser 0.87. ^{1}H-NMR (200 MHz, D_2O, Ref. DSS): δ 2.1-2.2 (m,2H), 2.55 (m, 2H), 3.83 (t, 1H, J=6.3 Hz), 3.87 (d, 2H J=5.2 Hz), 3.97 (s, 2H), 4.48 (t, 1H,J=5.2Hz). ^{13}C-NMR (50.1 MHz, D_2O, ref. TMS): 25.94, 31.24, 41.55, 53.80, 55.63, 61.21, 172.31, 173.54, 173.65, 175.00 ppm.

Synthesis of N-t-BOC-α-benzyl-γ-L-glutamyl-L-alanylglycine. Coupling of the active ester to L-AlaGly was accomplished by the procedure above with AlaGly as the dipeptide. For analytical purposes the protected peptide can be purified by reversed phase HPLC on a Dupont Zorbax ODS column (2.12 x 25 cm) eluted at 6 mL/min with 65% CH_3OH in 0.1 M acetic acid. Retention time was 24 min.

Synthesis of γ-L-glutamyl-L-alanylglycine. The protected alanyl peptide was deprotected and purified by anion exchange chromatography as described above for the seryl peptide. Amino Acid Analysis: Ala 1.00, Gly 0.98, Glu 1.00. ^{1}H-NMR (200 MHz, D_2O, ref. DSS) δ 1.37 (d, 3H, J=7.3Hz), 2.12 (m, 2H), 2.47 (m, 2H), 3.79 (t, 1H, J=6.3 Hz), 3.93 (s, 2H), 4.31 (quart. 1H, J=7.3 Hz). ^{13}C-NMR (50.1 MHz, D_2O, ref. dioxane) 17.45, 26.81, 32.04, 42.24, 50.51, 54.69, 174.29, 174.40, 175.30, 176.33 ppm.

Analytical Methods. Proton and ^{13}C-NMR spectra were taken on an IBM WP 200 SY spectrometer. Amino Acid compositions were determined after hydrolysis in 6 N HCl for 18 h at 100 °C on a Beckman analyzer. No correction was made for the decomposition of serine during hydrolysis.

Inhibition of Glutathione S-transferase. Kinetic studies of glutathione S-transferase were performed at 25 °C using 1-chloro-2,4-dinitrobenzene as the electrophile in either 0.1 M MOPS buffer (pH 7.3) or 0.1 M PIPES buffer (pH 6.5). Reactions were monitored at 340 nm ($\Delta\varepsilon$ = 9,600 $M^{-1}cm^{-1}$) (17) using a Perkin-Elmer 552 spectrometer. Glutathione solutions were prepared each day in MOPS or PIPES buffer, adjusted to the appropriate pH with NaOH under N_2 and stored on ice under a blanket of N_2 while in use. Solutions of 1-chloro-2,4-dinitrobenzene were prepared in CH_3CN. Inhibitors, GOH and GH were dissolved in MOPS or PIPES buffer and adjusted to the appropriate pH with NaOH. Reaction mixtures consisted of 0.9 mL buffer, and 25 μL each of solutions of appropriate concentrations of glutathione, 1-chloro-2,4-dinitrobenzene, inhibitor and enzyme. Dilute solutions of enzyme were found to be unstable even on ice so stock enzyme solutions were assayed at frequent intervals and prepared fresh when necessary. Spontaneous reaction was corrected for when necessary. Each reaction was run in triplicate. Initial velocity data were analyzed using the programs HYPER, LINE, COMP, and NONCOMP (18) where appropriate.

RESULTS AND DISCUSSION

Synthesis of Glutathione Analogues. The two tripeptide analogues can be assembled in a very simple three step sequence from commercially available

starting materials in good (65 - 70%) overall yield. Isolated yields of the protected tripeptides from the coupling reaction are highest if the pH of the reaction is maintained in the range of 8.5 to 8.7 and if, during work-up, the ethylacetate extract is back-extracted with water. It should be noted that it is unnecessary to purify the protected peptides by HPLC before deprotection except for analytical purposes since the peptides are purified by ion exchange chromatography in the final step.

Inhibition of Isozyme 3-3 by GOH and GH. At pH 6.5 isozyme 3-3 exhibited a K_m for GSH of 24 μM at saturating CDNB, a K_m for CDNB of 29 μM at saturating GSH and a turnover number of 12 s^{-1}. As shown in Figure 1 the oxygen analogue is a potent linear competitive inhibitor vs GSH with a K_I of 8.7 ± 0.7 μM. The desthio analogue, GH, is also a good competitive inhibitor of isozyme 3-3 (Figure 1) with a K_I of 80 ± 24 μM, about an order of magnitude higher than GOH. Interestingly, at saturating concentrations of the electrophile both GOH and GH are linear non-competitive (mixed type) inhibitors vs glutathione (data not shown). Linear replots of both slopes and intercepts suggest that EI_2

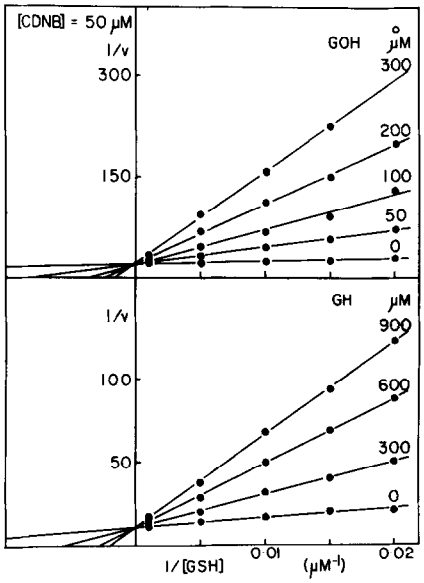

FIGURE 1. Inhibition of isozyme 3-3 by GOH (top) and GH (bottom) with fixed (50 μM) CDNB and variable (50 μM to 1 mM) glutathione at pH 6.5. Units of 1/v are $s \cdot \mu M^{-1}$. Solid lines are computer fits to the data points. Open data point was omitted from computer analysis.

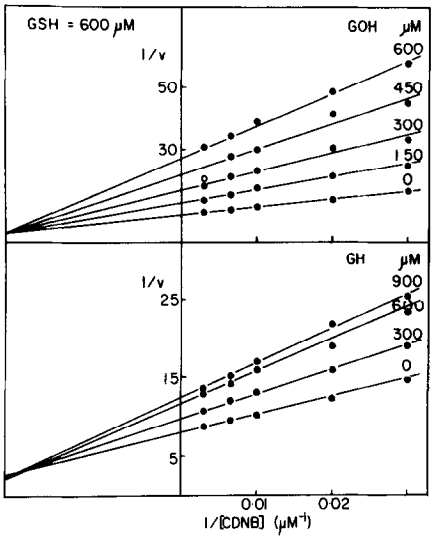

FIGURE 2. Inhibition of isozyme 3-3 by GOH (top) and GH (bottom) at fixed (600 μM) glutathione and variable (33 μM to 1 mM) CDNB. Units of 1/v are $s \cdot \mu M^{-1}$. Solid lines are computer fits to the data points with K_{IS} = 180 ± 26 μM K_{II} = 250 ± 23 μM for GOH and K_{IS} = 900 ± 80 μM and K_{II} = 1,700 ± 290 μM for GH. Open data point was omitted from computer analysis.

complexes are not the cause of the noncompetitive behavior. Although the exact molecular basis for the mixed-type inhibition vs GSH at saturating CDNB cannot be ascertained from the present data it appears that the peptides can, in the presence of saturating CDNB, bind in complexes such that they cannot be driven off by saturating GSH.

Inhibition of isozyme 3-3 by GOH and GH with CDNB as the variable substrate is clearly noncompetitive (mixed type) (Figure 2). This observation is consistent with either the steady-state random mechanism previously proposed (5) with 1,2-dichloro-4-nitrobenzene as the electrophile or an ordered mechanism in which glutathione adds first. It is clearly inconsistent with either an ordered or ping-pong mechanism where CDNB adds first. Were CDNB obliged to bind first GOH and GH would be expected to be uncompetitive inhibitors. This result is not surprising since glutathione binds to the free enzyme with high affinity (6) (Table I). Although glutathione S-transferases show characteristics of having random sequential kinetic mechanisms (5,8) it is clear that in a healthy cell where the intracellular concentration of glutathione is high relative to the dissociation constant for the E-GSH

TABLE I. Inhibition Constants for GOH and GH with isozymes 3-3 and 4-4 at pH 7.3 and low fixed concentration of CDNB

Isozyme	Peptide	K_I or K_D (μM)	$\delta\Delta G_{GSH}$[c] (kcal/mol)	$\delta\Delta G_{GOH}$[d]
3-3	GSH	7.0 ± 1.5[a]	-	-
	GOH	13.0 ± 2.7	0.4 ± 0.2	-
	GH	116 ± 6	1.7 ± 0.2	1.3 ± 0.2
4-4	GSH	(~10)[b]	-	-
	GOH	31.8 ± 3.0	(~0.7)[b]	-
	GH	134 ± 12	(~1.5)[b]	0.8 ± 0.1

[a]Dissociation constant from reference 6.

[b]Values estimated based on the presumption the K_D^{GSH} for isozyme 4-4 is of the same order of magnitude as for isozyme 3-3.

[c]Relative to GSH, $\delta\Delta G_{GSH} = RT \ln (K_I/K_D^{GSH})$.

[d]Relative to GOH, $\delta\Delta G_{GOH} = RT \ln (K_I^{GH}/K_I^{GOH})$.

complex, all of isozyme 3-3 is in the form of the E·GSH complex. For practical purposes the enzyme follows an ordered mechanism with GSH bound first.

Inhibition of isozyme 4-4 by GOH and GH. Isozyme 4-4 is the other homodimeric member of the family of isozymes derived from binary combinations of the closely related 3 and 4 subunits (13,19). At pH 6.5 the enzyme has a Km for GSH of 52 μM at saturating CDNB, a K_m for CDNB of 180 μM at saturating GSH and a turnover number of 9.5 s^{-1}. Both GOH and GH are linear competitive inhibitors of isozyme 4-4 *vs* glutathione at low (50 μM) concentrations of CDNB with K_I values of 35 ± 8.8 and 160 ± 10 μM, respectively. As with isozyme 3-3 both analogues are noncompetitive mixed type inhibitors with CDNB as the variable substrate where for GOH K_{IS} = 180 ± 10 μM and K_{II} = 360 ± 46 μM and for GH K_{IS} = 0.94 ± 0.08 mM and K_{II} = 1.9 ± 0.1 mM.

Recognition of Glutathione Analogues by Glutathione S-transferase. The thermodynamic dissociation constant for GSH and isozyme 3-3 is known (Table I) from equilibrium binding studies at pH 7.3. Under the assumption that the K_I

values for the two peptides at low concentrations of CDNB are reasonable approximations of their thermodynamic dissociation constants with the free enzyme, the contribution ($\delta\Delta G$) of the hydroxyl and sulfhydryl groups to the free energies of binding of GOH and GSH can be estimated. For this reason K_I values for the two peptides were determined at pH 7.3 for comparison with the K_D of GSH. The results, shown in Table I, suggest that the substitution of oxygen for sulfur in glutathione does not appreciably change the ability of isozyme 3-3 to recognize the peptide. However, removal of the hetero atom whether it be oxygen or sulfur causes a substantial loss (~1.5 kcal/mol) in the free energy of binding. Qualitatively similar results can be estimated for isozyme 4-4. Whether the differences in free energies of binding of the three peptides are due to differences in conformer populations of the peptides or to altered peptide-protein interactions or both remain to be determined.

Conclusions. The preliminary results presented here suggest that the two easily assembled peptide analogues of glutathione, γ-L-Glu-L-SerGly and γ-L-Glu-L-AlaGly are valuable molecules for the study of glutathione dependent enzymes. Their use as inhibitors of glutathione S-transferase and other enzymes is under active investigation.

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