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GAMMA-GLUTAMYLCYSTEINE: A SUBSTRATE FOR GLUTATHIONE *S*-TRANSFERASES*

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Abstract—A new high performance liquid chromatography (HPLC) method for the separation of γ-glutamylcysteine (GC) from glutathione (GSH) following derivatization with 1-chloro-2,4-dinitrobenzene (CDNB) was developed using a Vydac C_{18} column and an acetonitrile-trifluoroacetic acid gradient. When the derivatization of GC, GSH, cysteine, and cysteinylglycine was performed with GSH *S*-transferase, peak heights for the GC and GSH derivatives were accentuated markedly, suggesting that GC, like GSH, is an enzyme substrate. Subsequently, GC was found to be a substrate for five purified forms of rat hepatic GSH *S*-transferase. However, the K_m for GC was about 6–20 times higher than that for GSH. GSH was a competitive inhibitor of GC-CDNB conjugation, indicating that GC and GSH share the same binding site on the transferase. However, endogenous hepatic GC content in fed rats was only 5.8 ± 0.1 nmoles/g, three orders of magnitude lower than GSH. Thus, under normal circumstances, GC would not be expected to contribute to detoxification reactions catalyzed by the GSH *S*-transferases. Its weak interaction with the GSH site of the GSH *S*-transferases supports the role of the glycine moiety of GSH in enhancing this interaction.

Gamma-glutamylcysteine (GC) is an intermediate in the synthesis of glutathione (GSH) from cysteine. Although GSH participates in detoxification reactions catalyzed by GSH *S*-transferases, little is known about GC [1]. Habig *et al.* [2] reported previously that homoglutathione, gamma-glutamylcysteinylalanine, are as active as GSH in 1,2-dichloro-4-nitrobenzene conjugation using rat GSH *S*-transferase A and C, whereas L-cysteine, *N*-acetyl-L-cysteine and 2-mercaptoethanol are inactive. However, the activity with GC has not been tested. The lack of satisfactory methods for assessing contamination of GC by GSH and for separating reaction products of these two thiols has limited the study of GC. In the current study, we have examined the role of GC as a substrate for GSH *S*-transferase and have developed a high performance liquid chromatography (HPLC) technique for the separation of GC from GSH by derivatization with 1-chloro-2,4-dinitrobenzene (CDNB). The results indicate that GC substitutes for GSH as a substrate for GSH *S*-transferases but with lower affinity.

MATERIALS AND METHODS

Chemicals. GC disulfide was prepared from oxidized GSH (GSSG) with carboxypeptidase A according to the method of Strumeyer and Bloch [3]. Reduced GC was prepared from its disulfide by treatment with dithioerythritol (DTE). GSH, GSSG, L-cysteine, DTE, carboxypeptidase A and trifluoroacetic acid were obtained from the Sigma Chemical Co. (St. Louis, MO). L-Cysteinylglycine was reduced from L-cystinyl-bis-glycine obtained from Vega Chemicals (Tucson, AZ) with DTE. CDNB obtained from the Aldrich Chemical Co. (Milwaukee, WI) was recrystallized from ethanol-water before use. Acetonitrile was obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ). Epoxy-activated Sepharose 6B, chromatofocusing gel PBE 118 and Pharmalyte (pH 8 to 10.5) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other compounds used were readily available commercial products.

Animals. Male Sprague–Dawley rats (Hilltop Lab. Animals Inc., Scottdale, PA) weighing approximately 250 g were used for HPLC sample preparation and GST purification. Cytosol was prepared by homogenization of livers in 10 mM sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose (33%, w/v) followed by centrifugation at 100,000 *g* for 60 min.

HPLC methods. Thiol standards were prepared in 0.1 M sodium phosphate buffer (pH 6.5) and 5 mM DTE and derivatized with 4 mM CDNB using GSH *S*-transferases. Y protein (160 μg) [4] from rat liver cytosol was used as enzyme source for derivatization. The final reaction volume was 1 ml. To estimate the endogenous hepatic GC and GSH content, freshly prepared liver homogenates were precipitated in 5% trichloroacetic acid and mixed with DTE. Standards or liver extracts were derivatized with CDNB in the presence of GSH *S*-transferases after adjusting pH

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to 6.5. Derivatization was performed at 37° for 1 hr with gentle shaking, and then 2 ml methanol was added to 1-ml samples to stop the reaction. Under these conditions derivatization of GC and GSH was complete, whereas for cysteine and cysteinylglycine it was not. After blowing dry, samples were diluted with 1 ml water, and 250-μl aliquots were injected into the HPLC system at a final dilution of 1:160 of the original samples (standards or cytosol).

An Altex Scientific (Berkeley, CA) model 312 MP liquid chromatograph with a 210 sample injector and an Altex Hitachi 155-40 variable-wavelength detector was employed. All separations were performed on a Vydac C_{18} reversed phase column (5 μm, 250 × 4.6 mm) obtained from the Separation Group (Hesperia, CA). Solvent A was 0.1% trifluoroacetic acid, and B was 50% acetonitrile in 0.1% trifluoroacetic acid. Optimal separation was achieved with the following program: 1 min isocratic 30% B followed by a 15-min gradient from 30% to 50% B. Flow rate was maintained at 1 ml/min. All runs were performed at room temperature, and column effluent was monitored at 340 nm, which was determined to be the maximal absorbance for both GC and GSH conjugates (referred to as GC-DNB and GSH-DNB).

The concentration of GC standards was confirmed by determining amino acid concentration using a Beckman (Fullerton, CA) 119CL amino acid analyzer calibrated with Beckman amino acid hydrolysate standards. GSH concentrations in standards and liver were determined as previously described [5].

Thiol-disulfide status of samples. To ensure that derivatization with CDNB in the presence of DTE was both complete and that DTE completely reduced disulfides, an additional HPLC analysis was performed according to Reed *et al.* [6]. Samples incubated with DTE (5 mM) as above containing 50 μM cystine, GC disulfide, cysteinylglycine disulfide, or GSSG were treated as above (with or without CDNB). Then samples were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene and chromatographed on an amine-column as described by Reed *et al.* [6]. In the absence of CDNB, only the thiol forms were detected in this system, indicating complete reduction. In the presence of excess CDNB, no remaining thiol or disulfide forms were detected, indicating complete derivatization under our assay conditions.

Purification of rat liver GSH S-transferases. Purification was performed according to the method of Mannervik and Jensson [7]. Briefly, the purification procedure consisted of affinity chromatography of rat liver cytosol on *S*-octylglutathione bound to epoxy-activated Sepharose 6B followed by chromatofocusing with PBE 118 and pH gradient 11-8 using Pharmalyte. Enzyme activity was measured with CDNB as previously described [2]. Protein concentrations were determined by the method of Lowry *et al.* [8]. The purity of proteins was confirmed by discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis performed in vertical slab gels using the method of Laemmli [9]. The following transferases were isolated in homogeneous form: Y_aY_a, Y_aY_c, Y_bY_b, Y_bY_b, and $Y_{b'}Y_{b'}$. According to a recently described new nomenclature [10], these are GSH *S*-transferase 1-1, 1-2, 3-3, 3-4, and 4-4 respectively. Proteins were either used immediately following purification or stored at −70° in 30% glycerol, 1 mM GSH, then thawed and dialyzed before use.

Enzyme kinetic analysis. Using five pure forms of rat liver GSH *S*-transferase, enzyme kinetic studies were performed for GC and GSH conjugation. The production rate of GC- or GSH-DNB over a range of GC or GSH concentration and fixed CDNB (0.1 mM) was measured at pH 6.5, 37° spectrophotometrically at 340 nm using 3 ml reaction mixtures. Nonenzymatic reaction rates were subtracted from the enzymatic rates. Kinetic parameters were computed by non-linear least-squares fitting of the Michaelis–Menten model to the data. No enhancement of the rate of reaction of cysteine or cysteinylglycine with CDNB at 340 nm was observed with the GSH *S*-transferases.

Kinetic studies were also performed for GC conjugation using two other substrates, *p*-nitrobenzyl chloride (PNBC) and 3,4-dichloronitrobenzene (DCNB). Conjugation of GC with PNBC (0.5 mM) was carried out using GSH *S*-transferase C at pH 6.5, 37° in 3 ml reaction mixture. Absorbance of the conjugate was measured at 310 nm. Conjugation with DCNB (1.0 mM) was similarly performed using transferase C at pH 8.0, 37°, and the conjugate formed was monitored at 344 nm spectrophotometrically. Nonenzymatic (spontaneous) reaction rates were subtracted from the enzyme-catalyzed rates. The absorbance values selected represent λ_{max} for both GSH and GC with these substrates.

Kinetics of inhibition. To examine the nature of inhibition of GC by GSH, inhibitory kinetics were studied using GSH *S*-transferase-Y_aY_c. Different concentrations of GC (0–10 mM) were incubated at pH 6.5 with 0.1 mM CDNB using 0.6 μg of GSH *S*-transferase-Y_aY_c in the presence or absence of 0.15 mM GSH in a total volume of 1 ml. Reactions were carried out for 4 min, during which the rate of GC-DNB formation was linear. The reactions were stopped by adding an equal volume of 10% trichloroacetic acid. Samples were then analyzed by HPLC to quantitate GC-DNB (integration of peak areas). The data were expressed by the method of Hofstee [11], and kinetic parameters were determined by linear least-squares fit.

Determination of millimolar extinction coefficients for GC- and GSH-DNB conjugates. The millimolar extinction coefficients, *E*, for GC- and GSH-DNB conjugates were determined from the relationship

$$E = \frac{A}{(c)l}$$

where A = absorbance, (c) = concentration, and l = 1.0 cm path lengths. The GC- and GSH-DNB conjugate were purified by HPLC as described above. The absorbance A_{340} of the isolated fractions was measured, and the peaks were then subjected to acid hydrolysis for amino acid analysis (Beckman 6300 amino acid analyzer). A_{340} of the individual fractions was compared to their amino acid content. The calculated millimolar extinction coefficients were

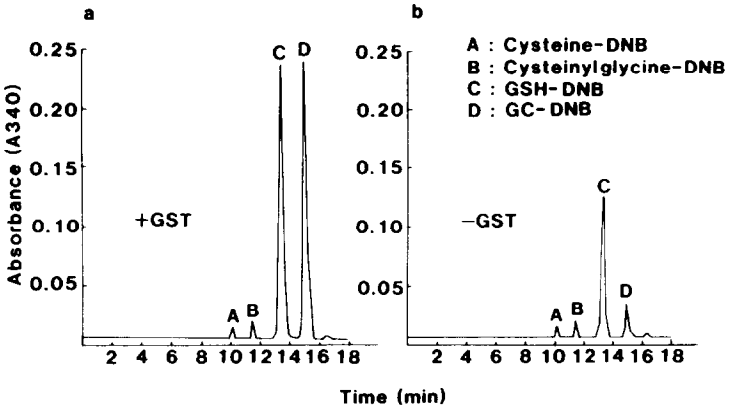

Fig. 1. HPLC separation of thiols derivatized with CDNB in the presence (a) or absence (b) of GSH S-transferases (GST). The ordinate represents A_{340} and the abscissa, retention time. The concentration of the standards used for derivatization was 25 μM.

(340 nm) 18.0 and 16.1 $mM^{-1} \cdot cm^{-1}$ for GC- and GSH-DNB respectively.

RESULTS

HPLC resolution and quantitation. Thiol standards derivatized with CDNB were clearly separated from one another and the retention times of cysteine, cysteinylglycine, GSH and GC conjugates were 10.2, 11.5, 13.4 and 15.0 min respectively (Fig. 1b). The resolution of GSH and GC conjugates with the Vydac column was not achieved with other C_{18} columns tested. When the derivatization was performed in the presence of GSH S-transferase, the peak heights of both GC-DNB and GSH-DNB derivatives were accentuated markedly, whereas those of cysteine and cysteinylglycine were not (Fig. 1a). Of note, the GC which we prepared showed no evidence of unreacted GSH using this HPLC method following reduction and derivatization. Standard curves for peak area versus concentration of GC and GSH were linear over the range of 10 pmoles to 60 nmoles (using 250 μl injection). The coefficient of variation of the assay using 1 mM GC standard was 4.8% (N = 4) and using 1.3 μM GC standard was 5.9% (N = 5). Using this HPLC method, endogenous GC concentration in liver was near the lower limit of detectability in fed rats (mean ± S.E.: 5.8 ± 0.1 nmoles/g, N = 4) compared to GSH content of 6.3 ± 0.2 μmoles/g. Of course, since the reaction of CDNB with cysteine and cysteinylglycine was not complete and nonenzymatic, this HPLC method could not be used to determine the tissue content of these thiols.

Enzyme kinetic studies. Figure 2 shows the effect of varying GC and GSH concentration on the rate of conjugation with CDNB catalyzed by GST-Y_aY_c and -Y_bY_b. A summary of the kinetic parameters using the five purified forms of rat liver GST is shown in Table 1. With each of the transferases, the K_m for GC was higher (6–20 times) than that for GSH, and the V_{max} for GC with CDNB was about half of that

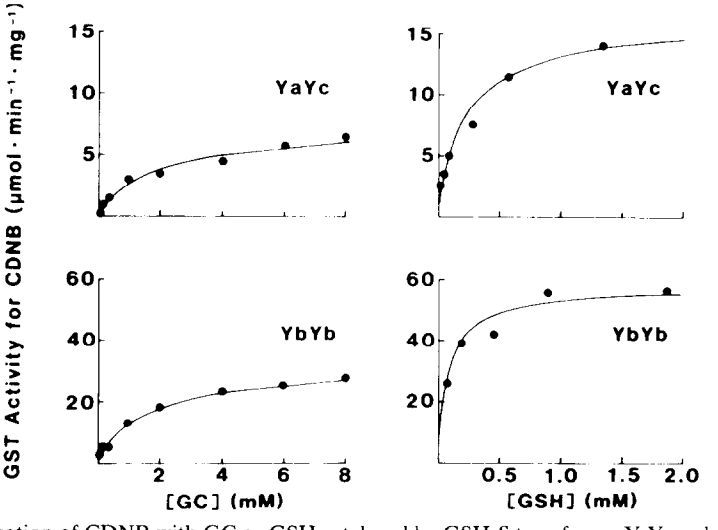

Fig. 2. Conjugation of CDNB with GC or GSH catalyzed by GSH S-transferase-Y_aY_c and -Y_bY_b. GSH S-transferase (GST) activity was measured with 0.1 mM CDNB and various concentrations of GC or GSH. The curves represent the nonlinear least-squares fits of the Michaelis–Menten model to the data.

Table 1. Kinetic parameters for five purified forms of rat liver GSH S-transferase with GC and GSH*

	K_m (mM)		V_{max} (μmoles·min^{-1}·mg^{-1})	
	GC	GSH	GC	GSH
Y_aY_a	0.97	0.15	6.0	14
Y_aY_c	1.83	0.23	7.4	16
Y_bY_b	1.53	0.08	32	57
$Y_bY_{b'}$	1.96	0.12	32	43
$Y_{b'}Y_{b'}$	0.99	0.10	26	44

* Enzyme activity was measured at 340 nm with CDNB (0.1 mM) as co-substrate. Kinetic parameters were determined by nonlinear least-squares fit. Freshly purified enzymes were used for these experiments. GC source contained no detectable GSH, and when the product of reaction with CDNB and GC was examined by HPLC, only GC-DNB was detected.

for GSH. To ensure that the results obtained with CDNB as substrate reflected other transferase substrates, the K_m values for GC and GSH with PNBC and DCNB were compared using transferase C ($Y_bY_{b'}$). Again, GC was found to be a co-substrate with a K_m 7- and 32-fold higher than that of GSH with PNBC and DCNB respectively.

Inhibition of GC-DNB formation by GSH. The data, depicted in the form of a Hofstee plot, showed that GSH is a competitive inhibitor of GC (Fig. 3). The K_i value for GSH (0.2 mM) corresponded closely to its K_m with GST-Y_aY_c (Table 1). The lower (by one-third) V_{max} in the inhibition study compared to that in Table 1 probably reflects loss of enzyme activity with storage at −70° over approximately 6 months.

DISCUSSION

A new HPLC method for the resolution of derivatized endogenous nonprotein thiols such as cysteine, cysteinylglycine, GSH and GC has been developed in the present study. With this method it has been possible to confirm the purity of our GC preparations, identify the reaction products, and examine the inhibitory kinetics of GC and GSH as substrates for GSH S-transferases. During development of this HPLC method, an enhanced peak height of the GC-CDNB conjugate in the presence of GSH S-transferases was noted, suggesting that GC is a substrate for GSH S-transferases. This suggestion was confirmed by kinetic studies in which GC was found to be a substrate for five purified forms of rat GST. Moreover, inhibition kinetics indicated that GC and GSH compete for the same site on GSH S-transferases. The K_m for GC, however, was about an order of magnitude higher than the K_m for GSH, indicating that GC is a substrate with a lower affinity for GSH S-transferases.

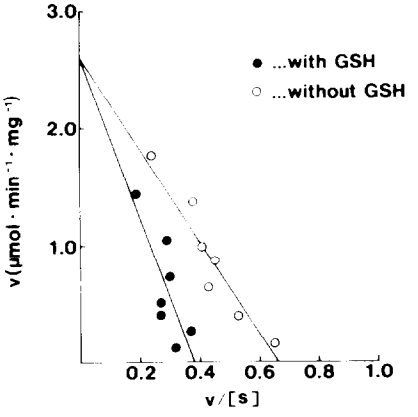

Fig. 3. Hofstee plot showing competitive inhibition of GC-DNB formation by GSH. Different concentrations of GC were incubated with 0.1 mM CDNB for 4 min using GSH S-transferase-Y_aY_c (0.6 μg/ml) as enzyme in the presence or absence of GSH (0.15 mM). The reaction products were quantitated by HPLC. The lines represent the linear least-squares fits to the transformed data.

To our knowledge GC is the first recognized endogenous thiol substrate for GSH S-transferases other than GSH in animals. Previously, homoglutathione, which is found in plants, was shown to be a substrate for rat GSH S-transferases [2]. Since GSH concentrations far exceed GC in normal liver and GSH S-transferases have higher affinities for GSH, one would predict an extremely small contribution of GC to hepatic metabolism of GSH S-transferase substrates. It has been suggested that the glycine moiety of GSH is critical for binding of GSH to the transferases [12, 13]. Our finding of a lower affinity for GC compared to GSH, although consistent with the importance of glycine for binding, indicate that the requirement for glycine is not absolute. However, when electrophilic substrates of GSH S-transferases preferentially deplete GSH, it is conceivable that the relative availability of GC for conjugation reactions may be greater and of potential importance. In addition, in glutathione synthetase deficient individuals, GC may be the dominant substrant for detoxification reactions catalyzed by GSH S-transferases.

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