GLUTATHIONE S-TRANSFERASE IN THE FORMATION OF CYANIDE FROM ORGANIC THIOCYANATES AND AS AN ORGANIC NITRATE REDUCTASE

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SUMMARY. Evidence is presented that glutathione S-transferases, a group of enzymes active in the formation of thioethers from glutathione and a large number of compounds with an electrophilic carbon atom, can also catalyze the formation of nitrous acid and oxidized glutathione from organic nitrate esters such as nitroglycerin. In addition, organic thiocyanates are cleaved by these enzymes to form cyanide and the respective asymmetric disulfide of glutathione.

Two additional roles have been found for the glutathione S-transferases (EC 2.5.1.18), a group of enzymes which we had previously isolated in homogenous form from rat liver and shown to catalyze the conjugation of GSH with a large number of compounds bearing an electrophilic site (1-3). Among the previously identified substrates were a variety of epoxides, sulfate esters, activated alkenes and halogenated and nitro derivatives (1-6); the product in each case is the corresponding thioether (Eq. 1).

$$RX + GSH \rightarrow RSG + HX \quad (1$$

Two reactions have been reported in which GSH is oxidized rather than participating in the formation of a thioether. Heppel and Hilmoe (7) had demonstrated that equivalent amounts of nitrite and oxidized glutathione were produced by a preparation of organic nitrate reductase from porcine liver acting upon the nitrate ester vasodilators, nitroglycerin or erythrityl tetranitrate (Eq. 2). In a somewhat analogous reaction, Ohkawa et al (8,9) reported that preparations from houseflies and mouse livers catalyze the liberation of cyanide and the formation of oxidized glutathione from organic thiocyanates (Eq. 3).

$$RCH_2ONO_2 + 2\ GSH \rightarrow RCH_2OH + HNO_2 + GSSG \quad (2$$

$$RCH_2SCN + 2\ GSH \rightarrow RCH_2SH + HCN + GSSG \quad (3$$

We report here the evidence for the glutathione transferases as catalysts in the reactions described by Equations 1 and 2, as well as in the liberation of cyanide from organic thiocyanates.

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MATERIALS AND METHODS. Gluthathione S-transferases A, B and C were homogenous preparations from rat liver (1). Glutathione reductase from yeast was purchased from Boehringer-Mannheim and ^{14}C-Glutathione (glycine-1-^{14}C), 12 μCi per μmole, was purchased from Amersham-Searle. Erythrityl tetranitrate was a gift from W. B. Brownell of Burroughs Wellcome Co. Nitroglycerin (glyceryl trinitrate), isosorbide dinitrate (1,4:3,6-dianhydrosorbitol 2,5-dinitrate), and ethylene glycol dinitrate were presented to us as dilutions of between 10 and 25% in lactose by T. Z. Ball and F. Cox of Atlas Powder Co.; the nitrates were extracted before use with 95% ethanol to produce a 0.1 M solution. The mixed disulfide of GSH and octyl mercaptan was prepared by the method of Eriksson (10) and purified by chromatography on Whatman 3MM paper in Solvent 1 (see below).

Assay methods for some of the substrates have been described (1). The standard assay system with organic thiocyanates included 0.75 mM of the thiocyanate, 1 mM GSH and enzyme in 0.6 ml of 0.1 M potassium phosphate at pH 7.5; control tubes without enzyme were included for every determination. The reaction was stopped after 10 min at 25° by addition of N-ethylmaleimide to 1.5 mM and cyanide was determined by a modification of the method of Epstein (11). The method of Ellman (12) was used for SH-group determination; where necessary, interference by cyanide was eliminated by bubbling helium through the acidified sample prior to assay. Oxidized glutathione was measured in a spectral assay with TPNH and glutathione reductase.

Descending paper chromatography was carried out on Whatman No. 1 with Solvent 1 (n-butanol:acetic acid:water; 12:3:5) or Solvent 2 (ethanol:water; 77:23). Synthetic octyl glutathionyl disulfide, GSH and GSSG migrated with R_f 0.77, 0.29 and 0.08, respectively, in Solvent 1 and R_f 0.72, 0.47 and 0.22, respectively, in Solvent 2.

RESULTS. The range of substrate specificity of homogeneous glutathione S-transferases A and B from rat liver is shown in Table 1 for several readily

Table 1

Specific activity for substrates with glutathione S-transferases A and B

Substrate (concentration)	Specific activity for transferase A	Specific activity for transferase B
	μmoles min^{-1} mg^{-1}	
1-Chloro-2,4-dinitrobenzene (1mM)	62	11
1,2-Epoxy-3-(p-nitrophenoxy)-propane (0.5 mM)	0.1	<0.006[a]
trans-4-Phenyl-3-buten-2-one (50 μM)	0.02	0.001
2-Nitropropane (20 mM)	0.012	0.005
Nitroglycerin (1 mM)	0.058	0.090
Erythrityl tetranitrate (1 mM)	0.76	0.36
Isosorbide dinitrate (1 mM)	0.015	0.004
Ethyleneglycol dinitrate (2 mM)	0.013	0.020
Ethyl thiocyanate (0.75 mM)	0.076	0.019
Octyl thiocyanate (0.75 mM)	0.12	0.047
Benzyl thiocyanate (0.75 mM)	0.59	0.084

[a] Lower limit of the assay at the highest enzyme concentration tested

Table 2

Partial stoichiometry for reactions catalyzed by glutathione S-transferase

Reaction type	Conditions				Change in concentration			
	Substrate (conc.)	GSH	Transferase (conc.)	Incubation time[a]	+GSSG	-SH	+HNO_2	+HCN
Equation	(mM)	mM	(μM)	min	μM			
1	2,3,5,6-Tetrachloronitrobenzene (0.05)	1	A(0.7)	10	1		28	
1	2-Nitropropane (20)	1	B(3.3)	10	3		50	
2	Nitroglycerin (1)	1	B(3.2)	10	290		250	
2	Erythrityl tetranitrate (1)	1	B(3.3)	10	240		250	
2	Erythrityl tetranitrate (1)	1	B(1.6)	5		160	80	
4	Octyl thiocyanate (0.6)	1	A(0.7)	15	- 3			43
4	Ethyl thiocyanate (0.75)	1	A(0.7)	15	3			32
4	Ethyl thiocyanate (10)	0.5	A(0.7)	10		145		130

[a] All reactions in 0.1 M potassium phosphate at pH 7.5

available nitrate esters and organic thiocyanates. For comparison, specific activities with some previously studied substrates for these enzymes (1,2) are included in the table. With nitroglycerin and 1mM GSH at pH7.5, the following kinetic parameters were obtained for transferases A, B and C, respectively: K_m = 6.5, 0.8 and 1.6 mM; catalytic constant = 15, 7 and 41 mol min^{-1}(mol enzyme)$^{-1}$ With ethyl thiocyanate under the same conditions: K_m = 5.3, 1.9 and 23 mM; catalytic constant = 35, 3 and 69 mol min^{-1}(mol enzyme)$^{-1}$.

The glutathione S-transferases catalyze the reaction of GSH with many nitro compounds to yield nitrite and the corresponding thioether. However, in the case of nitrate esters and organic nitrate reductase, the alcohol derivative of the parent compound and oxidized glutathione were found (7,13,14). These differences in utilization of GSH are reflected in the stoichiometry of the enzyme-catalyzed reactions (Table 2). Whereas the data are in accord with Equations 1 and 2, the reactions with the organic thiocyanates tested do not conform to the postulated Equation 3. Although cyanide was formed, only minor amounts of oxidized glutathione were detected. Rather, our findings are consistent with Equation 4.

$$RSCN + GSH \rightleftarrows RSSG + HCN \quad (4$$

Incubation of 330 μM GSH ($8.9x10^5$ dpm) and 4 mM octyl thiocyanate with 4 μg of transferase A in 100 μl of 0.1M potassium phosphate at pH 7.5 for 20 min. resulted in a new radioactive species, separable by paper chromatography in Solvents 1 and 2 at R_f 0.77 and 0.73, respectively. The new compound migrated to the same position as, and cochromatographed with, a synthetic preparation of the mixed disulfide, i.e. octylglutathionyl disulfide. Treatment of the radioactive enzyme product with 20 mM dithiothreitol and subsequent chromatography resulted in the absence of the new species and the identification of a radioactive compound migrating as GSH.

Despite evidence (1-3) for homogeneity of these enzymes on the basis of electrofocusing, sodium dodecylsulfate-gel electrophoresis and sedimentation equilibrium studies, the relatively low activities with nitrates and thiocyanates allows the possibility that these observations represent the effect of small amounts of contaminating enzymes. In a precipitin reaction with antibody prepared against transferase A, enzyme activity with 1-chloro-2,4-dinitrobenzene, nitroglycerin and ethyl thiocyanate was reduced in the same proportion at each concentration of antibody (Table 3).

DISCUSSION. The glutathione S-transferases function both as binding proteins for a large number of non-substrates such as bilirubin (15,16) and as catalytic agents for an equally large variety of substrates bearing dissimilar functional groups. To the group of substrates we must now add the

Table 3

Precipitation of three catalytic activities with antibody to glutathione S-transferase A

Gamma globulin dilution	Percent Residual Activity[a]		
	1-chloro-2,4-dinitrobenzene	Erythrityl tetranitrate	Ethyl thiocyanate
stock	0	0	5
1:2	28	23	33
1:4	85	82	92
1:8	95	113	99
1:16	100	100	96

Transferase A (13 µg in 10 µl) was incubated for 15 hours at 4° with 0.5 ml of phosphate buffered saline, pH 7.4, containing dilutions of the gamma globulin fraction of normal or anti-transferase A rabbit sera. Saturated ammonium sulfate (0.25 ml) was added to precipitate soluble antibody-antigen complexes; this concentration does not precipitate free transferase A. Following centrifugation, aliquots of the supernatant fluid were assayed for activity with the three substrates.

[a] Percent residual activity is defined as the percentage of activity remaining in the supernatant fluid after treatment with anti-transferase A gamma globulins as compared to treatment with an equal quantity of normal gamma globulins.

organic thiocyanates and nitrate esters, additions that appear to have heuristic value in suggesting a general mechanism for the reaction. The data point to GSH, the kinetically obligate first substrate (2,17), in the form of GS^-, as the active nucleophile attacking any one of a variety of electrophilic centers. Attack by GS^- on a carbon atom of the second substrate, bound to the enzyme, results in a thioether as product (Eq. 1). Reaction with alkyl thiocyanates is also considered a nucleophilic attack of GS^-, but one on the sulfur of the thiocyanate, producing an asymmetric disulfide; cyanide is the leaving group (Eq. 4). In the case of organic nitrate esters, the attack would be on the relatively electrophilic nitrate nitrogen forming an S-nitration product and alkoxide as the leaving group; the instability of the nitration product and the presence of a second molecule of GS^- would yield the observed products, i.e. GSSG and nitrous acid (Eq. 2).

In a more speculative vein, we note that the pK of the sulfhydryl of cysteine has been shown to vary by more than two orders of magnitude as a

function of neighboring ionizable groups (18) and suggest that a major role of the protein is in "activating" GSH by binding it in a manner favorable to ionization. Whether the second ligand is attacked by GS^- may then depend on its possessing a sufficiently electrophilic target. This idea serves as a working hypothesis and predicts that compounds with an even wider range of functional groups will act as substrates.

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