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THE GLUTATHIONE PEROXIDASE ACTIVITY OF GLUTATHIONE *S*-TRANSFERASES

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Summary

Glutathione transferases (RX:glutathione R-transferases, EC 2.5.1.18) B and AA were purified from rat liver to investigate the mechanism for their apparent GSH peroxidase activity (GSSG formation). Both transferases catalyze an overall reaction in which loss of cumene hydroperoxide is accompanied by a stoichiometric increase in GSSG. Inclusion of cysteamine, a thiol, results in a reduction of GSSG formation but has no effect on hydroperoxide loss. Cysteamine does not inhibit the transferase-catalyzed conjugation of GSH and 1-chloro-2,4-dinitrobenzene. Peroxidase reactions carried out in the presence of cyanide, another nucleophile, also result in a reduction of GSSG formation without altering the rate of cumene hydroperoxide loss; cyanide does not inhibit transferase activity with 1-chloro-2,4-dinitrobenzene. Both cysteamine and cyanide are capable of blocking GSSG formation in the non-enzymic oxidation of GSH by hydrogen peroxide without blocking H_2O_2 loss. These results are consistent with a mechanism for GSH transferases in which nucleophilic attack by GS^- on hydroperoxide results in a reactive intermediate, presumably the sulfenic acid of glutathione, GSOH.

$$GSH + ROOH \rightleftharpoons GSOH + ROH \quad (1)$$

This sulfenic acid then reacts non-enzymically with GSH to produce GSSG.

$$GSOH + GSH \rightleftharpoons GSSG + H_2O \quad (2)$$

The summing of Reactions 1 and 2 explains the observed stoichiometry. Cysteamine and cyanide can compete with GSH for the sulfenic acid in Reaction 2, thus reducing GSSG formation. This two-step sequence is also a likely pathway for disulfide formation by mild oxidation of thiols.

Introduction

Many mammalian tissues contain two enzymes capable of forming GSSG from GSH in the presence of organic hydroperoxides. These two enzymes were first detected as peaks of activity following gel filtration chromatography when fractions were assayed for glutathione peroxidase using cumene hydroperoxide as substrate [1,2]. Subsequently it was shown that these two peaks of activity represented the enzymes glutathione peroxidase (EC 1.11.1.9) and glutathione *S*-transferases (RX:glutathione R-transferases, EC 2.5.1.18) [3]. Glutathione peroxidase is a selenoprotein which catalyzes the overall reaction as follows:

$$ROOH + 2\ GSH \rightleftharpoons ROH + GSSG + H_2O \quad (1)$$

Some possible mechanisms for this enzyme have been reviewed [4].

Glutathione *S*-transferases are a group of proteins which catalyze the first reaction in mercapturic acid synthesis, the conjugation of GSH with a wide variety of hydrophobic substrates containing an electrophilic atom [5].

$$RX + GSH \rightleftharpoons RSG + HX \quad (2)$$

One reaction product of the nucleophilic attack by the thiolate anion of glutathione (GS^-) is a thioether. However, in some cases GSH is oxidized and does not participate in thioether formation [6]. GSSG can be detected in reactions with organic thiocyanates as substrates but not in stoichiometric amounts; however, when organic nitrate esters are acted upon by GSH transferases, GSSG is a reaction product. The reaction can be described as follows:

$$RCH_2ONO_2 + 2\ GSH \rightleftharpoons RCH_2OH + HNO_2 + GSSG \quad (3)$$

This apparent anomaly was solved when Keen et al. [7] demonstrated that the product of enzymic reaction was the unstable glutathione sulfenyl nitrite ($GSNO_2$), which subseqently reacted with another GSH to produce the observed products, GSSG and nitrite. They suggest that the GSH transferase mechanism involves nucleophilic attack by GS^- on some electrophilic atom (C, S, or N) in the substrate [7].

The formation of GSSG when GSH transferases act upon organic hydroperoxides (ROOH) could involve a similar mechanism in which GS^- attacks an electrophilic oxygen, resulting in the formation of the unstable sulfenic acid of glutathione.

$$ROOH + GSH \rightleftharpoons [GSOH] + ROH \quad (4)$$

In a reaction analogous to the sulfenyl nitrite [7], the sulfenic acid then reacts non-enzymically with GSH.

$$[GSOH] + GSH \rightleftharpoons GSSG + H_2O \quad (5)$$

However, the stoichiometry of the peroxidase activity of the GSH transferases has not previously been reported. Sulfenic acids may be intermediates in thiol oxidation to disulfides, although because of their reactivity sulfenic acids are not observed; however, they have been reported in certain proteins [8]. Many nucleophiles are capable of reacting with protein sulfenic acids. Among these are thiols [8,9], dimedone [10] and cyanide [11].

Cyanide has previously been shown to compete with GSH during the peroxidase assay of GSH transferases [12]. This observation can be explained by an effect of cyanide on either Reaction 4 or 5, since both require GSH.

The present paper describes evidence which supports the existence of an intermediate (tentatively GSOH) which is formed during the oxidation of GSH by cumene hydroperoxide catalyzed by purified rat liver GSH transferases B or AA. This intermediate is also formed during the non-enzymic oxidation of GSH by H_2O_2.

Materials and Methods

Sigma Chemical Co. was the source of GSH, GSSG, NADPH, xylenol orange, 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, cysteamine, cystamine, and fluoropyruvate. H_2O_2, KCN, and $FeSO_4 \cdot 7\ H_2O$ were purchased from Mallinkrodt. Cumene hydroperoxide and dimedone were products of Matheson Coleman Bell. Ethanolamine was from Eastman. Yeast glutathione reductase was purchased from Calbiochem and prepared for use as described previously [13]; the specific activity of the preparations averaged 147 units/mg. Purified ovine erythrocyte glutathione peroxidase was the generous gift of H.E. Ganther, Madison, WI.

Enzyme purification. Glutathione *S*-transferase B and AA were purified from female rat liver according to a modification of the method of Habig et al. [14], as described previously [3]. Purity was estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using 7.5% acrylamide gels [15]. Analysis of transferase B, purified through CM-cellulose chromatography, showed two bands; transferase AA samples resulted in a single band when 16 μg of protein (Lowry procedure) was applied. Both proteins were stored frozen in 0.5 mM GSH with 10% glycerol in 10 mM potassium phosphate (pH 6.7).

Enzymic assays. The transferase activity of GSH transferase was measured by thioether formation between GSH and either 1-chloro-2,4-dinitrobenzene (340 nm) or 1,2-dichloro-4-nitrobenzene (345 nm) according to Habig et al. [5]. The GSH peroxidase activity of the transferases was measured by monitoring GSSG formation (NADPH oxidation at 340 nm) in the presence of 4 μg GSH reductase, using conditions described previously [16], except that the final concentration of cumene hydroperoxide was increased to 1 mM. Peroxide solutions were standardized enzymically utilizing an excess of purified glutathione peroxidase and about 50 nmol of ROOH or H_2O_2.

Chemical analyses. H_2O_2 and cumene hydroperoxide were also determined by their ability to oxidize Fe^{2+} to Fe^{3+} followed by complexation with xylenol orange [17,18]. An aliquot, 25 or 50 μl, was added to 0.05 N H_2SO_4 (1 ml final) and then 2 ml of a solution containing 0.2 mM Fe^{2+} and 0.5 mM xylenol orange in 0.05 N H_2SO_4 was added. The absorbance was measured at 540 nm after 1 h using a Beckman model 24 spectrophotometer. For H_2O_2 $\epsilon = 2.7 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ in agreement with previous work [18]; however, for cumene hydroperoxide $\epsilon = 6.6 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, and attempts to reduce this with NaCl [18] were not successful. Of those peroxidase reaction adducts tested, only EDTA reduced the color formation. Therefore, separate standard curves were prepared when enzymic reactions were studied because EDTA was used in the prepara-

tion of the GSH reductase and GSH solutions.

Thiol content of GSH and cysteamine solutions was quantified by the method of Ellman [19]; cysteamine was alternatively measured by reaction with sodium fluoropyruvate [20].

Inhibitor studies. Details of the experimental conditions are given in the legends to the figures and tables. The reactions were conducted in cuvettes maintained at 25°C, and NADPH oxidation was recorded at 340 nm with a Beckman DU-2/Gilford 2000 spectrophotometer. Reactions were initiated with peroxide. Duplicate aliquots at zero time and at regular intervals were removed and added to 0.05 N H_2SO_4 on ice to terminate the reaction. The solution containing Fe^{2+} and xylenol orange was added following the last time point. These peroxidase assays were changed from the usual procedure [16] by using phosphate buffer and omitting additional EDTA. Studies employing cysteamine were carried out at pH 6.5 [7], but the pH was raised to 7.5 when cyanide was used to increase the CN^- concentration. GSSG was added to cuvettes following reactions in which inhibition was observed to insure that GSH reductase was not limiting. Some studies employing cyanide, cysteamine and the mixed disulfide of GSH and cysteamine were carried out using yeast GSH reductase and the GSH reductase assay conditions described in Ref. 13.

Mixed disulfide. The mixed disulfide of GSH and cysteamine was prepared by reacting GSH with cystamine (3 mol of cystamine/mol GSH) at pH 8.6 for 2 h at 37°C. The reaction mixture was acidified, concentrated and subjected to fractionation by paper electrophoresis in 0.05 M pyridinium acetate (pH 5.5) at 1200 V for 2 h. Larger samples were prepared by ion-exchange chromatography (Amberlite IR-45), paper electrophoresis and gel filtration on Sephadex G-10. Following electrophoresis and reaction with ninhydrin the mixed disulfide (blue-pink) has a slight cathodic migration and is well separated from cystamine-cysteamine (pink) and GSH-GSSG (blue).

Results and Discussion

Purification of transferases B and AA

The purification of rat liver GSH transferases B and AA was conducted several times with similar results. Chromatography on DEAE-cellulose, followed by Sephadex G-150, was effective in separating the selenoprotein glutathione peroxidase from GSH transferases and for equilibration prior to CM-cellulose [3]. Peaks of activity corresponding to transferase B have specific activities when assayed with 1-chloro-2,4-dinitrobenzene, ranging between 10.8 and 14.6 in good agreement with that reported by others [5] for pure enzyme, but two protein bands were observed when samples (20 μg) were subjected to SDS gel electrophoresis. Peaks of activity corresponding to transferase AA have specific activities ranging between 13.7 and 17.8 in agreement with previous work [14] and yield a single protein band following gel electrophoresis.

Further kinetic studies (Table I) were conducted on a preparation of transferase AA which had an activity ratio of 660 with 1-chloro-2,4-dinitrobenzene versus 1,2-dichloro-4-nitrobenzene. The kinetic constants, K_m and V, for this preparation determined with 1-chloro-2,4-dinitrobenzene are in good agreement with those reported by Habig et al. [14] for purified transferase AA and

TABLE I

KINETIC PROPERTIES OF RAT LIVER GLUTATHIONE TRANSFERASE AA

Activities were determined at 25°C in the presence of 1 mM GSH and 1 mM cosubstrate at pH 7.5 for peroxidase assays [16] and pH 6.5 for the transferase assay [5]. K_m and V were determined from double-reciprocal plots with GSH at 3 mM. Values in parentheses are from Habig et al. [14].

Substrate	Activity (units · mg^{-1})	K_m (mM)	V (units · μmol^{-1} enzyme)
1-Chloro-2,4-dinitrobenzene	18 (14)	0.4 (0.4)	916 (920)
Cumene hydroperoxide	11	0.4	930
tert-Butylhydroperoxide	3.2	3.0	715

indicate along with the electrophoresis data and low activity with 1,2-dichloro-4-nitrobenzene [14] that this sample is transferase AA. Kinetic constants were also determined for the peroxidase reaction (Table I). The peroxidase activity of transferase AA with either cumene or *tert*-butylhydroperoxide is similar to transferase B (unpublished results). Lawrence et al. [21] in studies with transferase B reported a K_m of 0.55 mM for cumene hydroperoxide and an apparent K_m of 2.3 mM for *tert*-butylhydroperoxide with GSH at 1.2 mM. Transferase AA has high maximum velocities with both hydroperoxides when compared to other substrates (Table I and Ref. 14).

Inhibitors of transferase AA and B

To investigate the mechanism for the peroxidase activity based upon the transferase reaction, conditions were employed to compare with previous work on transferase B in which 2-mercaptoethanolamine (cysteamine) was used [7]. Samples of GSH transferases B and AA were tested for inhibition by cysteamine in both the peroxidase and transferase assay. Similar results for both enzymes were obtained, but only the data for transferase AA are shown (Table II). Cysteamine inhibits the apparent peroxidase activity but not the transferase activity; ethanolamine, the oxygen analog, does not inhibit. Cysteamine appears to be a competitive inhibitor with GSH when data are plotted by the Dixon method ($1/v$ vs. inhibitor concentration). As reported previously [7], and verified in these studies, cysteamine does not interfere with the measurement of GSSG. When *tert*-butylhydroperoxide replaces cumene hydroperoxide as substrate, similar data are obtained.

Cyanide was reported to be an inhibitor of the peroxidase activity of GSH transferases [12]. It also reacts with a protein sulfenic acid [11]. It was therefore tested on transferase AA (Table II). In agreement with the studies using cysteamine, cyanide does inhibit the peroxidase reaction but not the transferase reaction. Cyanide appears to be a competitive inhibitor with GSH when tested with either transferase B or AA, which agrees with an earlier report [12].

A third compound, dimedone, which is also known to react with protein sulfenic acids [10], was studied in hopes of trapping the putative glutathione sulfenic acid (Reaction 5). However, dimedone inhibits both peroxidase and transferase activities (Table II) and is a competitive inhibitor of the hydrophobic substrates rather than GSH (data not shown). Ethanol, the solvent for dimedone, was run as a control, since at these levels it inhibits the

TABLE II

INHIBITORS OF GLUTATHIONE TRANSFERASE AA

Reactions were conducted at 25°C in 0.1 M potassium phosphate containing varying amounts of GSH transferase AA. Rates were recorded at 340 nm. Cumene hydroperoxide and 1-chloro-2,4-dinitrobenzene were employed as substrates for the peroxidase and transferase assays, respectively. Reactions under (A) were conducted at pH 6.5 with 1 mM GSH, and those under (B) at pH 7.5 with 0.2 mM GSH.

Adduct	Concentration (mM)	Units/mg	
		Peroxidase	Transferase
(A)			
Ethanolamine	1	11.1	18.1
Cysteamine	1	3.62	17.6
(B)			
None	—	10.0	15.5
KCl	10	10.2	15.4
KCN	10	4.17	15.6
Ethanol	*	8.37	14.5
Dimedone	10	6.00	9.31

* Ethanol, the solvent for dimedone, was added at 2.5% so that the final concentration was 3.5% or 4.0% for the peroxidase or transferase assays, respectively.

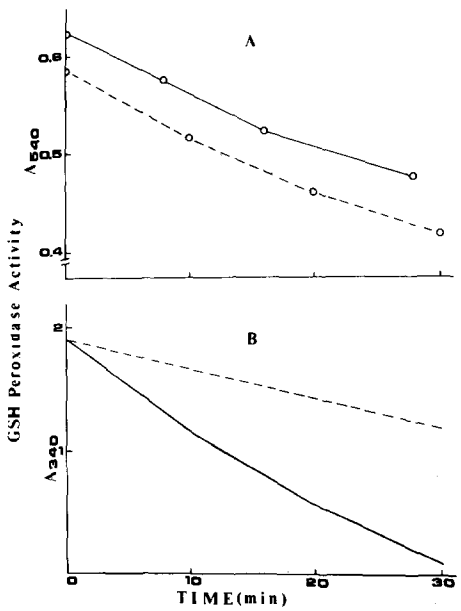

Fig. 1. Effect of cysteamine on the GSH peroxidase activity of GSH *S*-transferase B. The reaction was carried out at 25°C in a cuvette buffered with 0.1 M potassium phosphate (pH 6.5) containing 7.5 μg/ml of GSH transferase B, 4 μg/ml of GSH reductase, 0.3 mM NADPH, 1 mM cumene hydroperoxide and 1 mM GSH (———) or 1 mM GSH + 1 mM cysteamine (- - - - -). (A) At the times indicated (○) duplicate 25-μl aliquots were removed from the cuvette, initial volume 0.5 ml, and were analyzed for cumene hydroperoxide by incubating in the presence of Fe^{2+} and xylenol orange and measuring the A_{540}. (B) The loss of NADPH at 340 nm (GSSG formation) was continuously recorded except for those brief aliquot removals indicated in (A).

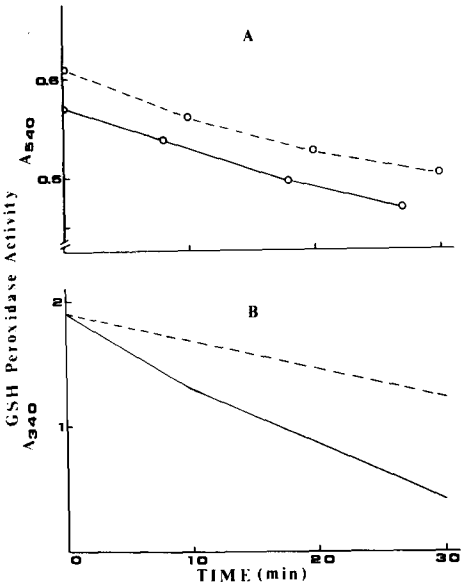

Fig. 2. Effect of cyanide on the GSH peroxidase activity of GSH *S*-transferase B. The reaction was carried out at 25°C in a cuvette buffered with 0.1 M potassium phosphate (pH 7.5) containing 7.5 μg/ml of GSH transferase B, 4 μg/ml of GSH reductase, 0.3 mM NADPH, 1 mM cumene hydroperoxide, and 0.2 mM GSH + 10 mM KCl (———) or 10 mM KCN (- - - - -). (A) At the times indicate (○) duplicate 25-μl aliquots were removed from the cuvette, initial volume 0.5 ml, and were analyzed for cumene hydroperoxide by incubating with Fe^{2+} and xylenol orange and measuring A_{540}. (B) The loss of NADPH (GSSG formation) at 340 nm was continuously recorded, except for those brief aliquot removals indicated in (A).

TABLE III

EFFECT OF CYSTEAMINE AND CYANIDE ON THE GSH PEROXIDASE ACTIVITY OF GSH TRANSFERASE B

Reactions were conducted at 25°C in 0.1 M potassium phosphate containing 4 μg/ml of glutathione reductase, 0.3 mM NADPH, 7.5 μg/ml of GSH transferase B (where indicated) and were initiated by adding cumene hydroperoxide (ROOH) to achieve 1 mM. Reactions under (A) were conducted at pH 6.5 with 1 mM GSH and 1 mM cysteamine (where indicated). Reactions under (B) were conducted at pH 7.5 with 0.2 mM GSH and either 10 mM KCN (i.e., CN^- = 0.15 mM) or KCl. Rates were continuously recorded at 340 nm for GSSG formation. At intervals two 25-μl aliquots were removed for duplicate analyses of residual ROOH by the xylenol orange procedure. Values are averages of at least two experiments. Net enzymic rates are reported for reactions containing transferase.

Reaction adducts	nmol · min^{-1} · ml^{-1}	
	GSSG formed	ROOH lost
(A)		
GSH	0.79	0.69
GSH + cysteamine	0.81	2.3
GSH + transferase	8.4	8.8
GSH + cysteamine + transferase	2.8	8.6
(B)		
GSH + KCl	0.71	0.81
GSH + KCN	0.39	0.5
GSH + KCl + transferase	6.9	6.8
GSH + KCN + transferase	3.0	6.4

peroxidase activity of transferase proteins (Table II and Ref. 3).

Since both cysteamine and cyanide appear to be competitive inhibitors of the peroxidase reaction and not the transferase reaction, yet both reactions are GSH dependent, further studies were carried out on the peroxidase reaction using transferase B. When the peroxidase reaction is followed by measuring the loss of cumene hydroperoxide (ROOH) (Reaction 4) concurrently with GSSG formation (Reaction 5) in the presence of cysteamine (Fig. 1) or cyanide (Fig. 2), an apparent disparity is evident. Both cysteamine and cyanide reduce GSSG formation (Figs. 1B and 2B) in agreement with the data obtained with transferase AA (Table II). However, ROOH loss does not appear to be affected (Figs. 1A and 2A). These experiments were repeated several times and the mean rates were calculated (Table III).

In the absence of transferase and inhibitors there is a slow oxidation of GSH to GSSG accompanied by an equimolar loss of ROOH (Reaction 1). The addition of transferase B increases the rate but the reaction stoichiometry holds (Table III, GSH + transferase and GSH + KCl + transferase). The stoichiometry of the reaction was also tested by utilizing purified ovine erythrocyte glutathione peroxidase in place of GSH transferase B. At pH 6.5 with 1 mM GSH and ROOH, glutathione peroxidase forms GSSG at a rate of 9.36 nmol $\cdot$ $min^{-1} \cdot ml^{-1}$ and consumes ROOH at a rate of 9.59 nmol $\cdot$ $min^{-1} \cdot ml^{-1}$.

When cysteamine is included in the peroxidase reaction of GSH transferase B, the rate of GSSG formation is inhibited; however, the loss of ROOH is not affected (Table III), as suggested by Fig. 1. These results are consistent with the conclusion that the cysteamine is competing with GSH for GSOH (Reaction 5) and does not inhibit the enzyme-catalyzed reaction (Reaction 4). The product of the competition reaction would be the mixed disulfide of GSH and cysteamine (GSSR). A spot corresponding to GSSR was observed after acidification and paper electrophoresis of a reaction mixture containing transferase, but further identification of GSSR was not made.

The addition of cysteamine with GSH in the absence of transferase results in a greater rate of ROOH loss (2.3 nmol $\cdot$ $min^{-1} \cdot ml^{-1}$) than GSSG formed (0.81 nmol $\cdot$ $min^{-1} \cdot ml^{-1}$). A plausible explanation is that the rate of peroxide breakdown by thiols depends on the thiolate anion rather than the thiol [22], and the thiol group of cysteamine is more acidic than that of GSH, a pK of 8 compared to 8.6—8.9 [23].

Further support for the mechanism of the peroxidase activity of GSH transferases was obtained by quantifying the inhibitory effects of cyanide (Table III) seen previously (Fig. 2). The addition of cyanide to the peroxidase reaction causes a depression in GSSG formation without a significant effect on ROOH loss (Table III). The data suggest that CN^- is competing with GSH (Reaction 5) and, like cysteamine, cyanide does not inhibit the enzyme (Reaction 4), contrary to an earlier report [12]. The product of the competing reaction with cyanide would be glutathione thiocyanate (GSCN). The data obtained utilizing both cysteamine and cyanide strongly suggest that the reaction catalyzed by the GSH transferases is described by Reaction 4 and that the observed stoichiometry (Reaction 1) is due to the sum of the transferase reaction (Reaction 4) and the non-enzymic reaction (Reaction 5). This mechanism is in agreement with that predicted by Keen et al. [7].

Non-enzymic oxidation of GSH

It has been suggested that the mild oxidation of thiols to disulfides might proceed via a sulfenic acid intermediate [8]. Therefore, a study of peroxide loss and disulfide formation in the presence of the nucleophiles cysteamine and cyanide is extended to reactions omitting GSH transferase and containing H_2O_2 in place of cumene hydroperoxide (Table IV). The rate of GSSG formation was equivalent to H_2O_2 loss at both pH 6.5 and 8.0 in the absence of cysteamine or cyanide, respectively (Table IV). Cysteamine reacts faster with H_2O_2 (8.3 nmol · min^{-1} · ml^{-1}) than does GSH (5.5 nmol · min^{-1} · ml^{-1}). This agrees with the data using cumene hydroperoxide in the absence of transferase (Table III). The combination of GSH + cysteamine results in a rate of H_2O_2 loss that is equivalent to their separate rates. However, GSSG formation is halved (Table IV). This suggests that the oxidation of GSH by H_2O_2 is a two-step reaction (Reactions 4 and 5) and that cysteamine can compete with GSH for GSOH in the second step (Reaction 5), yielding GSSR rather than GSSG.

The experiments with the nucleophile cyanide also support this conclusion (Table IV). In the presence of CN^-, the rate of H_2O_2 loss is twice as fast as the rate of GSSG formation. The slower H_2O_2 breakdown rate in the presence of CN^- (10.3 nmol · min^{-1} · ml^{-1}) compared to Cl^- (14.2 nmol · min^{-1} · ml^{-1}) may be due to a reduction in GSH concentration as GSCN is formed. The rate of H_2O_2 loss depends on the GSH concentration. GSH is initially 0.3 mM (Table IV).

GSH reductase inhibition

The interpretation of the inhibition data depends upon the unimpaired ability of GSH reductase to measure GSSG. Previous workers [7] using similar reaction conditions (an excess of GSH reductase) demonstrated that the presence of cysteamine did not interfere with GSSG measurement, and this is verified in the present study. Furthermore, when 0.04 μg GSH reductase is

TABLE IV

EFFECT OF CYSTEAMINE AND CYANIDE ON GSH OXIDATION BY H_2O_2

Reactions were conducted at 25°C in 0.1 M potassium phosphate containing 4 μg/ml of glutathione reductase and 0.3 mM NADPH; they were initiated by adding hydrogen peroxide to achieve 1 mM. Rates were recorded at 340 nm for GSSG formation. At intervals two 50-μl aliquots were removed for duplicate analyses of residual H_2O_2 by the xylenol orange procedure. Rates have been corrected for H_2O_2 breakdown in the absence of thiols. Reactions under (A) were conducted at pH 6.5 with 2 mM GSH and 2 mM cysteamine (where indicated). Reactions under (B) were conducted at pH 8.0 with 0.3 mM GSH and either 10 mM KCN (i.e., CN^- = 0.47 mM) or KCl.

Reaction adducts	nmol · min^{-1} · ml^{-1}	
	GSSG formed	H_2O_2 lost
(A)		
GSH	5.6	5.5
Cysteamine	0.2	8.3
GSH + cysteamine	2.7	14.4
(B)		
GSH + KCl	13.5	14.2
GSH + KCN	4.7	10.3

assayed with an excess of GSSG, neither 1 mM cysteamine or 1 mM cystamine inhibits NADPH oxidation, but cyanide (10 mM KCN at pH 7.6) does. However, in the presence of an excess of GSH reductase (4 μg compared to 0.04 μg) this amount of cyanide does not interfere with GSSG quantification.

The inhibition of the peroxidase reaction could also be explained if GSSG were consumed by cleavage with cysteamine or CN^-. The presence of an excess of GSH reductase argues against this possibility. Even though CN^- is known to attack GSSG [23], this reaction is slow compared to GSSG reduction in the presence of excess GSH reductase. The cyanide inhibition of GSH reductase described above is not altered by doubling the GSSG level. The reaction of cysteamine with GSSG (Reaction 6) favors GSSR formation [24], but GSSG is rapidly reduced in the presence of GSH reductase and NADPH [7].

$$RSH + GSSG \rightleftharpoons GSSR + GSH \quad (6)$$

The transferase-catalyzed formation of GSSG in the presence of cysteamine and GSH may come from GSH oxidation by peroxides (Reaction 1) but additionally can come from disulfide interchange reactions (Reactions 7 and 8).

$$RSSR + GSH \rightleftharpoons GSSR + RSH \quad (7)$$

$$GSSR + GSH \rightleftharpoons GSSG + RSH \quad (8)$$

Cystamine (RSSR) does form in the reaction between RSH and peroxides (unpublished). It reacts rapidly with GSH [24], but most likely non-enzymically because the polar disulfide will not bind to GSH transferase [25]. RSSR is not reduced by 4 μg of GSH reductase when tested at 2 mM. Reaction 8 is normally rate limiting in the interchange reaction [24] but was used in the present study to quantify GSSR when coupled with GSH reductase and NADPH (not shown).

GSSR is a substrate for procine erythrocyte GSH reductase [26] and thus a potential error arises (Reaction 9), since NADPH oxidation is the basis for measuring GSSG formation.

$$GSSR + NADPH + H^+ \rightleftharpoons GSH \pm RSH + NADP^+ \quad (9)$$

Therefore, GSSR was purified and studied with yeast GSH reductase *. Although the activity of the reductase is less than 0.5% with GSSR compared to GSSG at 0.5 mM, a portion of the NADPH utilization may be via Reaction 9. However, the NADPH oxidation rate in the presence of cysteamine most likely comes from GSSG reduction by GSH reductase (Reaction 10).

$$GSSG + NADPH + H^+ \rightleftharpoons 2\,GSH + NADPH \quad (10)$$

The source of the GSSG is derived from a mixture of Reactions 1, 8 and 7 + 8. It is diffult to predict how effective cysteamine is in competing with GSH in

* The yeast enzyme was assayed at 0.5 mM GSSG and 0.5 mM GSSR. The rate with GSSG is 2.77 μmol/min and with GSSR is 0.012, based on 15 μM reductase. When expressed in this manner, these rates are directly comparable to work done on the rat liver enzyme [27]. GSSR yields a rate similar to the mixed disulfide of GSH and cysteine (0.050 μmol/min). From a double-reciprocal plot an apparent K_m of 3 mM for GSSR was obtained for yeast GSH reductase.

Reaction 5 due to the complex nature of side reactions. The amount of inhibition of that reaction (less GSSG) is most certainly underestimated.

Transferases as peroxidases

The mechanism for the peroxidase activity of the transferase proteins is described by Reactions 4 and 5. In vivo the transferases are saturated with GSH and, therefore, the levels and cellular location of endogenous hydroperoxides dictate whether or not the transferases can reduce hydroperoxides and compete with glutathione peroxidase [3]. Some evidence suggesting a role for the transferases in peroxide metabolism comes from the work of Lawrence et al. [21] who reported increased transferase activities in the livers of selenium-deficient rats which have low levels of glutathione peroxidase. Furthermore in some tissues the peroxidase activity from transferase is much greater than from glutathione peroxidase [3,28]. Guinea pig liver, for example, has no detectable selenium-dependent glutathione peroxidase activity [28]. When rat livers, depleted of glutathione peroxidase by selenium deficiency, were perfused with *tert*-butylhydroperoxide, GSSG was released into the perfusate [29]; there was no release when H_2O_2 was infused. H_2O_2 is not a substrate for the transferases [2]. These observations imply that the transferases can operate in the intact liver. However, GSH transferases have not been tested with membrane-derived hydroperoxides, the likely in vivo substrate.

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