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PURIFICATION AND PROPERTIES OF RAT LIVER MITOCHONDRIAL GLUTATHIONE PEROXIDASE

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Summary

Glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) was purified from rat liver mitochondria. The enzyme was shown to be pure by polyacrylamide-gel electrophoresis and to contain multiple forms that differed in charge. Selenium was specifically associated with the enzyme. The enzyme was inhibited by iodoacetic acid and iodoacetamide in an unusual pattern of reduction by sulfhydryl compounds and pH dependency. The mitochondrial and cytoplasmic forms of the enzyme were compared, and an explanation of the inhibition patterns is offered.

Introduction

Glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) catalyzes the reaction 2 GSH + ROOH → GSSG + ROH + H_2O [1], where ROOH is any of several hydroperoxide substrates [2,3] and ROH is the corresponding alcohol. The enzyme is found in a wide variety of organisms and tissues [4—9]. Rotruck et al. [10] first identified rat erythrocyte glutathione peroxidase as a selenium-containing enzyme, and selenium has since been found to be a constituent of several other glutathione peroxidases [7,11—13]. The enzyme functions to protect cellular membranes from oxidative damage [14—16]. Liver, which is a major site for detoxification and is thus exposed to high levels of oxidants, has high glutathione peroxidase levels [4]. Within rat liver, $\frac{2}{3}$ to $\frac{3}{4}$ of the cellular glutathione peroxidase is found in the cytoplasm and the remaining $\frac{1}{4}$ to $\frac{1}{3}$ is in the mitochondria, where the enzyme is located within the matrix [17,18]. In addition to generalized protection from lipid peroxidation, it probably functions to protect the inner mitochondrial membrane, which cannot be reached by the cytoplasmic enzyme, from hydrogen peroxide produced during mitochondrial respiration [19]. Glutathione peroxidase is identical with the Contraction Factor I necessary for reversal of glutathione-induced, high

amplitude swelling of mitochondria [20]. Mitochondrial glutathione peroxidase has recently been hypothesized to also have a modulating role in mitochondrial substrate oxidations [21].

Recently, several glutathione peroxidase have been purified and partially characterized [7,8,12,22], including the rat liver cytoplasmic enzyme [9,12], and the selenium-containing moiety of the rat liver cytoplasmic enzyme has been shown to be selenocysteine [23]. However, several mitochondrial enzymes have been found to be significantly different from their cytoplasmic counterparts [24—26], and mitochondrial glutathione peroxidase has not previously been purified and characterized except to show that 60% of the mitochondrial selenium content is associated with glutathione peroxidase [27].

This paper presents a purification of rat liver mitochondrial glutathione peroxidase and compares some of its properties with those of its cytoplasmic counterpart and those of other glutathione peroxidases. Partial characterization of the active site is also described.

Materials and Methods

Materials. Retired male breeder Sprague-Dawley rats were obtained from Simonsen Laboratories, Inc. Sephadex G-100 and G-150 were purchased from Pharmacia Fine Chemicals; DEAE-Agarose and hydroxyapatite from Bio-Rad Laboratories; Amicon membranes and filtration device from Amicon Corporation; cumene hydroperoxide and *t*-butyl hydroperoxide from Polysciences, Inc.; 5,5′-dithiobis-2-nitrobenzoic acid from Aldrich Chem. Co.; hydrogen peroxide from Mallinckrodt Chemical Works. Sodium [^{75}Se]selenite (400 μCi/ml) was obtained from New England Nuclear. All other reagents and buffers were obtained from Sigma Chemical Co.

Enzyme and protein assays. Glutathione peroxidase was assayed by a modification of the procedure of Paglia and Valentine [28]. A 0.1-ml sample of enzyme was mixed with 1.5 ml assay mixture at 37°C; then, 0.05 ml 1 mg/ml cumene hydroperoxide was added to initiate the reaction. The final reaction mixture contained 0.25 mM glutathione, 0.2 mM cumene hydroperoxide, 0.12 mM NADPH, 1 unit/ml glutathione reductase (1 μmol NADPH oxidized/min), and 0.091 mM EDTA in 50 mM Tris · HCl (pH 7.6). Activity was measured by the disappearance of NADPH at 340 nm in a Beckman DB spectrophotometer. Activity of glutathione peroxidase is expressed either as μmol NADPH oxidized/min or as change in absorbance/min at 340 nm (the change in absorbance is directly proportional to enzyme units).

For the sulfhydryl substrate specificity and nucleotide inhibition experiments, glutathione peroxidase was assayed by determination of residual sulfhydryl. A modification of the procedure of Sedlak and Lindsay [29] utilizing Ellman's reagent, 5,5′-dithiobis-2-nitrobenzoic acid [30], was used. A 1 ml reaction volume was incubated for 5 min at 37°C, after which the reaction was stopped by addition of 1 ml 10% (w/v) trichloroacetic acid. Subsequent addition of 2.0 ml 0.4 M Tris buffer/0.02 M EDTA (pH 8.9) adjusted the pH to 8.3. The mixture was incubated for 10 min at 25°C following addition of 0.05 ml of 0.01 M Ellman's reagent in absolute methanol. Absorbance at 412 nm was

noted immediately to determine the concentration of residual glutathione or other sulfhydryl compounds.

Glutamate dehydrogenase was assayed by the procedure of Leighton et al. [31] and glucose-6-phosphate dehydrogenase by the procedure of Langdon [32]. Protein was assayed either by the procedure of Miller [33], using bovine serum albumin as standard, or by measurement of absorbance at 225 nm.

Enzyme purification. Mitochondria from rat liver were isolated by an adaptation of the method of Chappell and Hansford [34]. Rats were killed by decapitation. The livers were removed into 5 vols. cold isolation medium of 0.25 M sucrose/1 mM EDTA/3.4 mM Tris (pH 7.4). Livers were finely minced with scissors and homogenized in a mechanically-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 600 × *g* for 10 min to remove whole cells and debris. This supernatant was centrifuged at 10 000 × *g* for 7 min to pellet the mitochondria, which were resuspended in isolation medium and spun at 10 000 × *g* for 7 min. This wash was repeated and the mitochondrial pellet was used for further purification.

Mitochondria were made 0.2% in Triton X-100, stirred for 30 min at 4°C, and centrifuged at 30 000 × *g* for 1 h. The supernatant was decanted as the mitochondrial matrix. HCl was added to adjust the matrix sample to pH 5.0. The sample was allowed to stand in the cold for 1 h, and then centrifuged at 15 000 × *g* for 15 min. The supernatant was decanted and NaOH added to adjust the pH to 7.4. This acid-treated sample was made 50% in acetone at −20°C and immediately centrifuged at 15 000 × *g* for 15 min. The pellet was resuspended in 10 mM Tris buffer (pH 7.6) and nonsoluble materials were sedimented by centrifugation at 15 000 × *g* for 15 min and discarded.

The resuspended acetone precipitate was chromatographed through a 5 × 100 cm Sephadex G-100 column and eluted with 10 mM Tris buffer (pH 7.6). The enzymatically active fractions were concentrated by high pressure filtration through an Amicon PM 30 membrane. The concentrated sample was applied to a DEAE-Agarose column (2.5 × 40 cm) and washed with 3 mM Tris buffer (pH 8.0). A linear gradient of 0—0.2 M NaCl in the same buffer eluted the enzyme near the beginning of the gradient. The enzymatically active fractions were pooled and concentrated as previously described and then applied to a Sephadex G-150 column (2.5 × 100 cm) and eluted in 10 mM Tris buffer (pH 7.6). The enzymatically active fractions were applied to a hydroxyapatite column (1.5 × 15 cm), which was washed with 0.05 M potassium phosphate (pH 7.6). The enzyme was then eluted in 0.1 M potassium phosphate (pH 7.6), and the enzymatically active fractions were pooled.

Gel electrophoresis. Polyacrylamide gels for electrophoresis were prepared according to Ornstein [35] and Davis [36]. Gels were stacked at pH 6.7 and electrophoresed at pH 8.9 with a phenol red dye front. Gels were stained for protein with Coomassie Blue. Enzymatic activity in gels was determined by the following procedure. After the gels were soaked for 1 min in 0.02 M glutathione/0.01 M cumene hydroperoxide, they were removed and put into 50% saturated nitroprusside, 8% (w/v) NH_4OH for 10 s. The gels were rinsed and immediately examined for areas of no staining, which were indicative of glutathione peroxidase activity.

Selenium in mitochondrial glutathione peroxidase. Rats were injected sub-

cutaneously with 0.1 ml [^{75}Se]sodium selenite (400 μCi/ml) 4 days prior to killing. Livers were excised and mitochondria were prepared as described. Matrix material obtained from the isolated mitochondria as described above was applied to a DEAE-Agarose column (2.5 × 40 cm), which was washed with 3 mM Tris buffer (pH 8.0) prior to application of a linear gradient of 0—0.2 M NaCl in buffer.

Radioactivity counting. ^{75}Se was detected in a Packard Model 3002 Tri-Carb scintillation counter that had been converted for gamma-ray detection with a Bicron NaI vial. Counting efficiency was approx. 16%.

Substrate specificity and nucleotide inhibition of purified mitochondrial glutathione peroxidase. Hydroperoxide substrate specificity was determined by substitution of other hydroperoxides for cumene hydroperoxide in the NADPH-coupled assay. Sulfhydryl substrate specificity was determined with Ellman's reagent on solutions containing 0.2 mM cumene hydroperoxide, 0.01 units purified mitochondrial glutathione peroxidase, and 0.35 mM sulfhydryl compound in 1 ml 50 mM Tris/0.1 mM EDTA (pH 7.6).

Nucleotide inhibition was determined similarly to sulfhydryl substrate specificity with the addition of appropriate nucleotide. Glutathione was the sulfhydryl substrate used.

DEAE-agarose chromatography of mitochondrial and cytoplasmic glutathione peroxidase. Liver homogenate was prepared as described above. An aliquot of homogenate was centrifuged at 11 000 × *g* for 12 min, and the supernatant was then centrifuged at 100 000 × *g* for 90 min. This supernatant was decanted and used as the cytoplasmic fraction. Another aliquot of homogenate was prepared by mitochondrial isolation and matrix material preparation as decribed above.

Samples were applied to a DEAE-Agarose column (2.5 × 40 cm), washed with 3 mM Tris buffer (pH 8.0), and eluted with a linear gradient of 0—0.2 M NaCl in buffer.

Reactions of alkylating reagents with mitochondrial glutathione peroxidase. Mitochondrial matrix material was prepared as described above. The acetone precipitate was then prepared, and the resuspended sample was applied to a Sephadex G-150 column (2.5 × 100 cm) and eluted with 10 mM Tris buffer (pH 7.6). The enzymatically active fractions were pooled, adjusted to pH 8.0, and applied to a DEAE-Agarose column (2.5 × 40 cm). This column was washed with 3 mM Tris buffer (pH 8.0) and the enzyme was eluted with a linear gradient of 0—0.2 M NaCl in the same buffer. The enzymatically active fractions were pooled and the buffer was changed to 50 mM Tris/0.1 mM EDTA (pH 7.6). This semi-purified sample with a specific activity of 60 000 units/mg was used in the following experiments.

Inhibition of glutathione peroxidase by iodoacetic acid and iodoacetamide was determined by the following procedure. All solutions and reagents were in 50 mM Tris/0.1 mM EDTA (pH 7.6). A 0.05-ml aliquot of either 10 mM glutathione or 4 mM hydrogen peroxide was added to 0.95 ml enzyme sample. After preincubation for 20 min at 32°C, 0.05 ml of either 20 mM iodoacetic acid or 20 mM iodoacetamide was added and incubation was continued at 32°C. After addition of inhibitor, 0.05-ml aliquots were removed at appropriate time intervals and assayed for glutathione peroxidase activity.

Effects of non-glutathione sulfhydryl compounds were determined by substituting either cysteine, dithioerythritol, or mercaptoethanol for glutathione during the preincubation. Effects of pH were determined using a buffer of 50 mM Tris/25 mM sodium phosphate/25 mM sodium citrate/0.1 mM EDTA (pH 7.6) during the preincubation with glutathione and adding hydrochloric acid to obtain the desired pH just prior to addition of iodoacetic acid. Procedures were otherwise as described above.

Results

Enzyme purification. The mitochondria isolated as described in Methods were assayed for activity of glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme not found in mitochondria. Mitochondrial samples consistently contained 1% or less of the total glucose-6-phosphate dehydrogenase activity of the homogenate and were therefore considered free of cytoplasmic contamination.

Since the glutathione peroxidase of mitochondria is localized in the matrix [17], a nonionic detergent, Triton X-100, was used to disrupt the mitochondrial membranes and to obtain the matrix material. Adjustment with acid to pH 5 precipitated much non-glutathione peroxidase protein. An acetone precipitation was used in the purification in order to purify further the enzyme and also to concentrate it prior to column chromatography.

Four chromatographic columns were employed in sequence: Sephadex G-100, DEAE-Agarose, Sephadex G-150, and hydroxyapatite. A representative purification scheme and results are summarized in Table I.

Polyacrylamide gel electrophoresis of purified mitochondrial glutathione peroxidase showed one broad band when gels were stained for enzymatic activity. Coomassie Blue stain for protein showed several distinct bands in the region corresponding to the activity stain. No protein was visible elsewhere on the gel.

Selenium in mitochondrial glutathione peroxidase. When mitochondrial glutathione peroxidase was isolated from rats injected with [^{75}Se]sodium selenite, the mitochondrial matrix contained 70% of the total ^{75}Se of the whole mitochondria, which is consistent with previous results [27]. Upon DEAE-Agarose

TABLE I

PURIFICATION OF RAT LIVER MITOCHONDRIAL GLUTATHIONE PEROXIDASE

Details are described in Results.

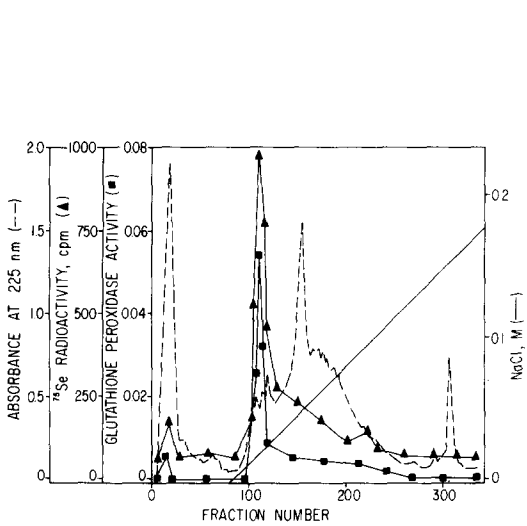
Sample	Total protein (mg)	Specific activity (μmol NADPH oxidized/min per mg protein)	Total activity (μmol NADPH oxidized per min × 10^{-3})	Yield (%)	Purification (-fold)
Mitochondria + Triton	1277	0.196	250	100	1.0
Matrix	1028	0.195	200	80	0.99
Acid treated	602	0.275	166	66	1.4
Acetone precipitate	63	0.995	62.8	25	5.1
Sephadex G-100	15	1.96	29.4	12	10
DEAE-Agarose	4.7	3.3	15.7	6	17
Sephadex G-150	1.1	6.4	6.7	3	33
Hydroxyapatite	0.033	123.4	4.0	1.6	631

chromatography, the ^{75}Se from mitochondrial matrix cochromatographed with glutathione peroxidase in a manner that was much more specific than that for other proteins (Fig. 1). Glutathione peroxidase appeared to account for the greatest part of the mitochondrial matrix selenium.

Substrate specificity of mitochondrial glutathione peroxidase. Substrate specificity of the purified mitochondrial glutathione peroxidase was determined for several sulfhydryl and hydroperoxide substrates. Glutathione was the only effective sulfhydryl substrate. The enzymatic reaction rates with cysteine, mercaptoethanol, and dithioerythritol were less than 5% of that with glutathione. Hydrogen peroxide was the most effective hydroperoxide substrate, but *t*-butyl hydroperoxide and cumene hydroperoxide had 86% and 88%, respectively, of the enzymatic reaction rate with hydrogen peroxide.

Nucleotide inhibition of mitochondrial glutathione peroxidase. Assay of purified mitochondrial glutathione peroxidase by the Ellman's reagent procedure was performed in the presence of different nucleotides, and inhibition was observed. Inhibition by 0.26 mM NADPH was 55%, by 1.0 mM NADH was 25%, by 1.0 mM adenosine was 18%, and by 1.0 mM nicotinamide was 2%.

DEAE-Agarose chromatography of mitochondrial and cytoplasmic glutatione peroxidase. Fig. 2A shows that chromatography profile obtained when freshly homogenized tissue was applied to the DEAE-Agarose column and eluted with a linear salt gradient. There was a major enzyme peak that did not bind to the column and a smaller peak that eluted at the beginning of the salt



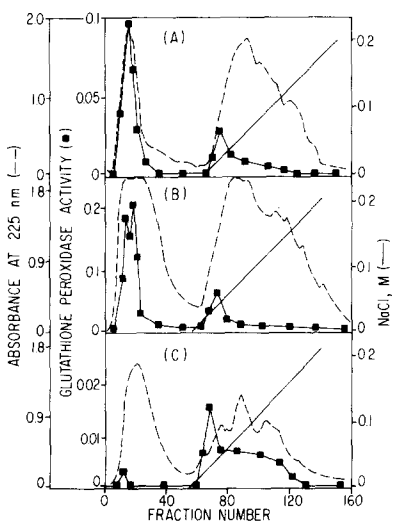


Fig. 1. DEAE-Agarose chromatograph of mitochondrial matrix material from rats injected with [^{75}Se]-sodium selenite. Sample was applied to the column, washed with 3 mM Tris buffer (pH 8.0), and eluted with a linear gradient of 0—0.2 M NaCl in buffer. ------, protein absorbance at 225 nm; ■———■, glutathione peroxidase activity expressed as ΔA at 340 nm/min per 0.1 ml; ▲———▲, γ-radioactivity of ^{75}Se; ———, NaCl molarity.

Fig. 2. DEAE-Agarose chromatograph of rat liver fractions. Sample was applied to the column, washed with 3 mM Tris buffer (pH 8.0), and eluted with a linear gradient of 0—0.2 M NaCl in the same buffer. ------, protein absorbance at 225 nm; ■———■, glutathione peroxidase activity expressed as ΔA at 340 nm/min per 0.1 ml; ———, NaCl molarity. (A) Homogenate; (B), cytoplasm; (C), mitochondria.

gradient. A similar chromatography of freshly prepared cytoplasm, shown in Fig. 2B, indicates a pattern similar to that for the fresh homogenate. The cytoplasm sample did not contain mitochondrial contamination since there was no significant activity of a mitochondrial marker enzyme, glutamate dehydrogenase. No change was observed in chromatography of the cytoplasm sample after it was incubated in Triton X-100 and centrifuged as in the normal procedure used for preparation of mitochondrial samples.

DEAE-Agarose chromatography of freshly prepared mitochondrial matrix produced the profile in Fig. 2C. The dominant peak appeared at the beginning of the salt gradient, and the peak not binding to the column was much smaller. Assays for glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme, showed insignificant activity.

Reactions of alkylating reagents with mitochondrial glutathione peroxidase. Partially purified glutathione peroxidase was utilized in the following experiments. Neither cyanide nor azide had an effect on the enzymatic reaction rate or on the NADPH-coupled assay for glutathione peroxidase. Column chromatography on Sephadex G-150 and on DEAE-Agarose showed that the enzyme sample contained no selenium that was not coincident with glutathione peroxidase. Neither iodoacetic acid nor iodoacetamide had an effect on the NADPH-coupled assay for glutathione peroxidase activity.

Addition of iodoacetic acid or iodoacetamide significantly inhibited glutathione peroxidase that had been preincubated with glutathione (Fig. 3). Iodoacetic acid produced much quicker inhibition than iodoacetamide. Enzyme preincubated with hydrogen peroxide proved to be relatively resistant to both reagents. Inhibition was irreversible in all reactions. When a sample of purified cytoplasmic enzyme [13] was assayed under these conditions it showed an identical inhibition pattern.

Glutathione peroxidase that was preincubated with cysteine, dithioerythritol,

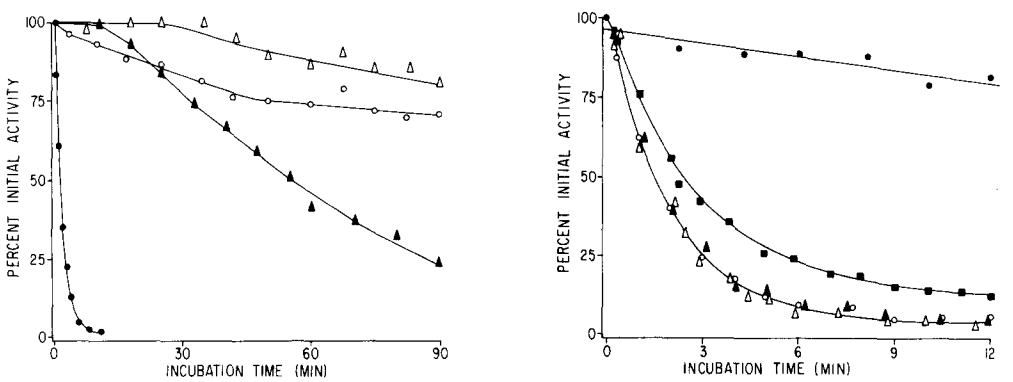


Fig. 3. Inhibition of glutathione peroxidase by alkylating reagents. Procedures are described in Materials and Methods. Iodoacetic acid reaction with reduced enzyme (●) and with oxidized enzyme (○). Iodoacetamide reaction with reduced enzyme (▲) and with oxidized enzyme (△).

Fig. 4. Inhibitory effect of iodoacetate on glutathione following prior incubation of enzyme with sulfhydryl compounds. Procedures are described in Materials and Methods. Pre-incubation with cysteine (■), dithioerythritol (○), mercaptoethanol (▲), glutathione (△), and hydrogen peroxide as oxidized control (●).

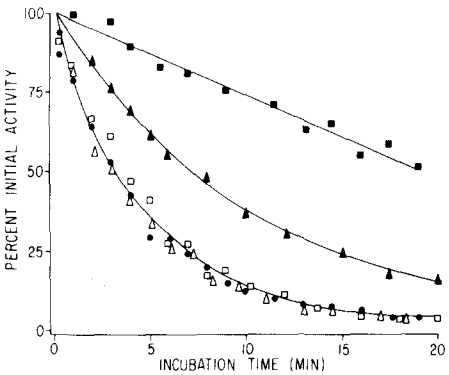


Fig. 5. Effect of pH on iodoacetic acid inhibition of reduced glutathione peroxidase. Procedures are described in Materials and Methods. Iodoacetic acid inhibition at pH 7.6 (●), 7.0 (△), 6.0 (□), 5.0 (▲), and 4.0 (■).

or mercaptoethanol in place of glutathione also proved to be highly susceptible to iodoacetic acid inhibition (Fig. 4). Dithioerythritol and mercaptoethanol were as effective as glutathione in sensitizing the enzyme to inhibition while cysteine was slightly less effective.

Inhibition of glutathione peroxidase by iodoacetic acid at different pH levels generated the pattern of inhibition shown in Fig. 5. The rates of inhibition were the same at pH 7.6, 7.0, and 6.0. Inhibition at pH 5.0 was significantly slower, and that at pH 4.0 was slower yet. Zero-time reaction rates were significantly different at the different pH levels; Fig. 5 shows only the relative changes in rate with pH.

Discussion

The procedure for purification of mitochondrial glutathione peroxidase described above and in Table I yielded purified enzyme. Gel electrophoresis showed protein stains only in regions coincident with enzyme activity although there were always several discrete protein stains for each broad activity stain. These were presumed to be isoenzymes of glutathione peroxidase as described by Stults et al. [13] and Chiu et al. [12] for the rat liver and lung cytoplasmic enzymes, respectively. The purified mitochondrial enzyme (Table I) had a final specific activity of 123 units/mg protein, which is significantly lower than the purified rat liver cytoplasmic enzyme specific activity of 278 units/mg protein [13] or the purified rat lung cytoplasmic enzyme specific activity of 173 units/mg protein [12], but higher than that of the purified human erythrocyte specific activity of 50 units/mg protein [8]. Preincubation of the mitochondrial enzyme with GSH could not activate it sufficiently to account for these differences. Because of the low yields obtained by this purification procedure, mitochondrial glutathione peroxidase was purified several times and variability in the value of the final specific activity was noted; the highest value obtained was 200 units/mg protein and the lowest was 110 units/mg protein. Since purified samples of differing specific activities all showed absence of extraneous

protein on gel electrophoresis, the variability in final specific activity is attributable to different amounts of enzyme inactivation and protein denaturation occurring in each sample.

The discovery of Rotruck et al. [10] that rat erythrocyte glutathione peroxidase was a selenium-containing enzyme provided an explanation for previously puzzling relationships between these two cellular components. Levander et al. [27] showed that selenium in rat liver mitochondrial glutathione peroxidase accounted for about 60% of the mitochondrial selenium. Fig. 1 confirms that the mitochondrial glutathione peroxidase has a specificity for selenium like that of the cytoplasmic enzyme [13], and that it contains a major part of the total mitochondrial selenium. The elution profile of mitochondrial glutathione peroxidase on Sephadex G-150 is similar to that of the cytoplasmic enzyme. The substrate specificity of mitochondrial glutathione peroxidase is similar to that reported for the enzyme from other sources [2,12,37,38], since it is highly specific for glutathione and it has a wide specificity for hydroperoxide. The mitochondrial enzyme is sensitive to inhibition by nucleotides similarly to the porcine erythrocyte enzyme [5]. Inhibition by the adenosine moiety (18% inhibition) and not by the nicotinamide moiety (2% inhibition) is also similar to inhibition of the porcine enzyme by these compounds [5]. The extent of inhibition is much stronger for the rat liver mitochondrial enzyme than for the porcine erythrocyte enzyme since 0.26 mM NADPH produced 55% inhibition of the mitochondrial enzyme but only 30% inhibition of the porcine enzyme.

This strong inhibition by NADPH means that kinetic measurement of glutathione peroxidase activity cannot be performed by the NADPH-coupled assay of Paglia and Valentine [28]. Alternative assay procedures have been developed (see refs. 39 and 40 and Materials and Methods).

The elution profile on DEAE-agarose of freshly prepared rat liver homogenate showed two peaks of glutathione peroxidase activity, and differences between the elution profiles of the cytoplasmic and mitochondrial enzymes were readily apparent (Fig. 2). At pH 8, the mitochondrial glutathione peroxidase was more negatively charged than the cytoplasmic enzyme. This difference appears to represent different charge isomers from those described for the cytoplasmic enzyme from liver [13] and from lung [12]. It thus appears that rat liver cells contain at least two distinct forms of glutathione peroxidase that differ in their charge characteristics. Only the more negatively charged form is found in the mitichondria while both forms are found in the cytoplasm.

A serious drawback of the purification procedure is that only very small amounts of purified enzyme are obtained. The yield was often less than 1.5% of the initial glutathione peroxidase total activity. This makes characterization of the enzyme difficult, and so a compromise was established between purity and quantity. Mitochondrial matrix sample purified through acetone precipitation and Sephadex G-150 and DEAE-agarose chromatography was used for the following inhibition studies. This semi-purified enzyme had a specific activity about one-half that of the completely purified enzyme, and the preparation had no selenium present other than that associated with the glutathione peroxidase. Proteins known to interfere with the assay of glutathione peroxidase were not contaminants in the sample since they are all cytochromes or heme-

containing proteins and neither cyanide nor azide had an effect on the reaction rate.

Inhibition of glutathione peroxidase by either iodoacetate or iodoacetamide occurred only when the enzyme was previously treated with glutathione and not when it was previously treated with hydrogen peroxide (Fig. 3). These results are in accord with those of ref. 37 for bovine erythrocyte enzyme, and the same results were also found by us for the rat liver cytoplasmic enzyme. Glutathione is both a substrate of the enzyme and a general reducing agent; likewise hydrogen peroxide is both a substrate and a general oxidizing agent. Sulfhydryl compounds other than glutathione sensitized the enzyme to inhibition (Fig. 4). These compounds are reducing agents but none are substrates for glutathione peroxidase. The enzyme apparently must be in a reduced form in order to be inhibited by alkylating reagents. Cysteine was not as effective an enzyme reducer as other compounds tested. Cysteine has the lowest pK for the sulfhydryl group of any of the tested compounds, and it could possibly react with the iodoacetic acid faster than the other sulfhydryl compounds. This would produce a lower effective iodoacetic acid concentration, which would slow down the inhibition.

Recently, rat liver mitochondrial glutathione peroxidase has been shown to utilize non-glutathione sulfhydryl compounds in the enzymatic reaction when glutathione is present also (ref. 40 and Forstrom, J.W. and Tappel, A.L., unpublished data). The specificity of the enzyme for glutathione is apparently for only one of the two sulfhydryls required in the reaction cycle, while it has wide specificity for the other sulfhydryl compound. This is consistent with the above results, which indicate that the enzyme is reduced by any of several sulfhydryl compounds and, in fact, may indicate that the enzyme has a low specificity for the first sulfhydryl utilized. Therefore, the high specificity for glutathione is as the second sulfhydryl compound required for a complete reaction cycle.

Iodoacetic acid reacts with several types of protein functional groups but at a different pH for each type [41]. Thus, sulfhydryls are reactive above pH 7, histidines above pH 5.5, amines above pH 8.5, and methionines in the range of pH 2—8.5. The pH dependency pattern of Fig. 5 apparently does not correspond to any of these functional groups. A reactive histidine might show this pH profile, but it should not react so quickly [42]. An active site cysteine sulfhydryl group made "active" in the same manner as "active" serines might account for the results if the sulfhydryl pK were lowered sufficiently.

The unusual inhibition patterns of mitochondrial glutathione peroxidase in response to alkylation reagents and the similar behavior of the cytoplasmic enzyme imply a common catalytic site for these two enzymes. The critical residue in inhibition of rat liver cytoplasmic glutathione peroxidase is selenocysteine, which has been identified as the sole selenium-containing moiety and as the active site residue in the enzyme derivatized by iodoacetic acid [23]. It is thus proposed that the active site of the mitochondrial enzyme also contains selenocysteine.

The selenol group of selenocysteine has a pK of 5.24 [43], which would produce the pH dependency of iodoacetic acid inhibition seen in Fig. 5. The presence of selenocysteine in the enzyme active site also would explain the

inhibition of reduced but not oxidized glutathione peroxidase by these alkylating reagents. Reduced glutathione peroxidase apparently has a free selenol group, R-SeH, which is susceptible to alkylation. Upon reaction with hydrogen peroxide, this group is oxidized to produce a species that cannot be derivatized with iodoacetic acid or iodoacetamide. Subsequent reduction regenerates the susceptible free selenol group. This reduction can be accomplished by any of several sulfhydryl reducing agents in addition to the substrate glutathione as discussed above.

The rat liver cytoplasmic and mitochondrial glutathione peroxidases would thus appear to have identical active sites, i.e., selenocysteine, and yet still to be different charge isomers on DEAE-Agarose chromatography. The differences between the two enzymes are thus believed to be minor and are probably made up of slight charge variations in the amino acid content in noncritical portions of the protein.

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