Identification of the Catalytic Site of Rat Liver Glutathione Peroxidase as Selenocysteine†

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ABSTRACT: A procedure was developed to isolate ^{75}Se-labeled rat liver glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9) at 30–50% purity with 20–30% yields in 4–5 days. Using these preparations of glutathione peroxidase, the selenium moiety in the enzyme was identified as selenocysteine by derivatizing the seleno group with either iodoacetate or ethylenimine in the intact protein, hydrolyzing the protein with 6 N HCl, and cochromatographing the ^{75}Se-labeled products with known standards. Techniques employed were anion-exchange chromatography, cation-exchange chromatography, gel-permeation chromatography, two-dimensional thin-layer chromatography, and automated amino acid analysis. The selenocysteine moiety was identified as the catalytic site in glutathione peroxidase by specifically labeling the enzyme with [^{14}C]iodoacetate on the ^{75}Se-labeled selenium atom and fractionating the ^{14}C, ^{75}Se-labeled derivative after acid hydrolysis. It was concluded that the reduced form of glutathione peroxidase contains the selenocysteine selenol (-SeH) at the catalytic site.

Glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9) functions to catalyze the reaction: ROOH + 2GSH → ROH + GSSG + HOH, where GSH is reduced glutathione,[1] GSSG is oxidized glutathione, and ROOH is any of a wide variety of hydroperoxides (Mills, 1959; Little and O'Brien, 1968). This reaction also serves to stop further peroxide initiation of lipid peroxidation and thereby protects the integrity of the cell (Flohé and Zimmerman, 1970; Tappel, 1974; Combs et al., 1975). Glutathione peroxidase from several mammalian sources is known to contain selenium (Se) specifically (Rotruck et al., 1973; Flohé et al., 1973; Oh et al., 1974). Probably Se exerts its trace element and antioxidant properties via its incorporation into mammalian glutathione peroxidase (Hoekstra, 1975). Glutathione peroxidase is the only known mammalian enzyme with a requirement for Se, although Se is known to be required in several bacterial enzyme systems, such as the glycine reductase of *Clostridium sticklandii* (Turner and Stadtman, 1973), in which the form of Se was identified recently as Se-Cys (Cone et al., 1976), and formate dehydrogenase of *Escherichia coli* and several other anaerobic bacteria (Pinsent, 1954; Andreeson and Ljungdahl, 1973; Enoch and Lester, 1972, 1975). The form of Se in the mammalian enzyme has been unknown until now. This paper reports that the Se in glutathione peroxidase is in the form of Se-Cys and that Se-Cys is the catalytic moiety in the enzyme. Se in the reduced form of the enzyme probably exists as the selenol (-SeH).

Experimental Procedure

Assays. ^{75}Se was counted on a Packard Model 5110 Modumatic Auto-Gamma spectrometer with an efficiency of about 30%. ^{3}H and ^{14}C were determined with a Beckman CPM-100 scintillation counter using Aquasol as the scintillation fluid. In all experiments where ^{3}H or ^{14}C was measured by liquid scintillation in the presence of ^{75}Se, the data were corrected for the ^{75}Se contribution. The relative efficiencies of ^{75}Se detection were determined for the gamma scintillation spectrometer and the liquid scintillation counter. After ^{75}Se determination of a sample on the gamma counter, the ^{75}Se contribution to liquid scintillation counting was calculated and subtracted from the net cpm. Relative ^{75}Se efficiencies were determined for each experiment as well as the absolute efficiency for ^{3}H (using a $^{3}H_2O$ standard) and ^{14}C (using a [^{14}C]toluene standard) on the liquid scintillation counter. With internal standards of $^{3}H_2O$, [^{14}C]toluene, and [^{75}Se] H_2SeO_3, it was determined that internal quenching did not affect the liquid scintillation data. Se was analyzed by the fluorometric method of Whatkinson (1966). Cyclohexane was used for extractions instead of decalin. Selenite and Se-Met were used as standards, and both gave the same fluorescence intensity per nanogram of Se.

A microfluorescamine method (Udenfriend et al., 1972) was used for determination of amino groups. A 50-μL aliquot of each sample was added to 0.5 mL of 0.2 M sodium borate buffer, pH 9.0, and 0.1 mL of 0.3 mg of fluorescamine/mL in dioxane or acetone was then added to the sample being mixed on a vortex mixer. Fluorescence (390-nm excitation and 475-nm emission) was measured immediately with an Aminco-Bowman spectrophotofluorometer.

Glutathione peroxidase activity was assayed according to Stults et al. (1977).

Preparation of Glutathione Peroxidase. Partially purified glutathione peroxidase was prepared routinely by a method similar to that of Stults et al. (1977). Ten 300–350-g male Sprague-Dawley rats were used for each purification. Three days prior to decapitation, two of the rats were given subcu-

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[1] Abbreviations used are: Se-Cys, selenocysteine; Se-Met, selenomethionine; SeCys(cm), carboxymethylselenocysteine; Se-Cys(Ae), aminoethylselenocysteine; [^{75}Se] product (Cm), purified ^{75}Se-labeled product from the acid hydrolysis of carboxymethylated [^{75}Se]glutathione peroxidase; [^{75}Se]products([^{14}C]Cm), ^{14}C,^{75}Se-labeled products from the acid hydrolysis of [^{14}C]carboxymethyl[^{75}Se]glutathione peroxidase; [^{75}Se]product(Ae), ^{75}Se-labeled product from the acid hydrolysis of aminoethylated [^{75}Se]glutathione peroxidase; GSH, reduced glutathione; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; DEAE, diethylaminoethyl.

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taneous injections of 50 μCi of $H_2{}^{75}SeO_3$ (104 mCi/mg). The livers were homogenized in ice-cold 0.25 M sucrose, 1 mM EDTA (1 g of liver/5 mL of sucrose). After straining through cheesecloth, the homogenate was acidified to pH 5.3 with GSH (free acid). The final GSH concentration was ca. 10 mM. The supernatant was adjusted to a pH of 7.6 with 100 mM Tris, heat treated, and cooled, and the protein precipitated with acetone. The protein precipitate was resuspended manually in 50 mL of 10 mM Tris, 5 mM GSH, 0.1 mM EDTA at pH 7.5 in a glass and Teflon homogenizer. The sample was centrifuged, and the supernatant was chromatographed on a Sephadex G-100 column in 10 mM Tris, 0.1 mM EDTA, pH 7.6. Pooled Sephadex G-100 fractions (255 mL) were made 3 mM in mercaptoethanol and treated batchwise with 40 mL of a DEAE-cellulose slurry (1 part settled gel to 1 part buffer, v/v). DEAE-cellulose was equilibrated with 10 mM Tris, 0.1 mM EDTA at pH 7.5. The supernatant was concentrated by ultrafiltration and twice chromatographed on DEAE-agarose columns in 10 mM Tris, 0.1 mM EDTA, pH 7.6. The enzyme sample was made 3 mM in mercaptoethanol before concentration by ultrafiltration. Glutathione peroxidase was retarded on these columns and eluted after the major protein fractions but without application of a gradient. The glutathione peroxidase was then concentrated and precipitated by dialysis against 4 M potassium phosphate, pH 7.1, and stored as the precipitate at 4 °C for use in future experiments. About 20–30% of the glutathione peroxidase activity and 12–15% of the ^{75}Se originally in the homogenate were recovered in the partially purified enzyme. Glutathione peroxidase activity is stable for several months when stored as the phosphate precipitate.

Preparation of Carboxymethylselenocysteine and Aminoethylselenocysteine. Se-Cys([^{3}H]Cm) was generated from the reaction of iodo[^{3}H]acetic acid and Se-Cys as described by Fletcher (1975). Determination of purity and identification of the Se-Cys([^{3}H]Cm) were assessed by noting the absence of peaks in the ultraviolet and visible spectra obtained on a Cary 118C spectrophotometer (except for a minor shoulder at 255 nm due to the seleno ether) and by elemental analysis, which yielded values of 26.72% C, 4.13% H, 6.06% N, 36% Se (theoretical = 26.56% C, 4.01% H, 6.20% N, 35% Se). Chromatography of the sample on AG1-X8, AG50W-X8, DEAE-agarose and automated amino acid analysis all showed single peaks in the regions predicted by theory.

Aminoethylselenocysteine was prepared by the reaction of ethylenimine with selenocysteine as described by Raftery and Cole (1963). The final residue appeared to be an oily material. Cavallini et al. (1955) reported that *S*-aminoethylcysteine also appeared to be an oily material at first and was only obtained in crystalline form after an extensive recrystallization procedure. The aminoethylated Se-Cys product contained 30% Se by weight (theoretical = 37% for monohydrochloride) and apparently still contained residual water and HCl. The product showed a single brown ninhydrin spot on thin-layer chromatography.

Mass Spectrometry. The *N*-acetyl, *O*-methyl derivatives of Se-Cys(Ae) and Se-Cys(Cm) were prepared for mass spectrometry. N-Acetylation of alkaline samples was performed according to the method of Morris et al. (1971). O-Methylation was then performed by allowing the sample to remain in 0.1 mL of methanol at room temperature for 10 days, after which each compound appeared to be a white, semicrystalline material. Mass spectrometry was done by direct probe analysis on a Finnigan 3200 GC–MS coupled to a Finnigan 6000 data-acquisition system. The *N*-acetyl, *O*-methyl derivative of Se-Cys(Ae) was identified by the Se isotope pattern at 310 mass units (molecular ion). Other Se isotope patterns were observed at 251 (loss of methylated carboxyl), 208 (loss of methylated carboxyl and acetyl groups) and 192 mass units (loss of methylated carboxyl, *N*-acetyl, and a hydrogen). The *N*-acetyl, di-*O*-methyl derivative of Se-Cys(Cm) showed the Se isotope pattern of the molecular ion at 297 mass units. Also observed was a peak associated with the *N*-acetyl, mono-*O*-methyl derivative. Although mass spectrometry alone was not sufficient for definitive identification of the compounds, these mass unit values were consistent with predicted values for these structures.

Carboxymethylation of Glutathione Peroxidase. An aliquot of about 1.5 mg of the phosphate-precipitated [^{75}Se]glutathione peroxidase was dissolved in 1 mL of 0.5 mM GSH and incubated at 37 °C for 10 min; 100 μL of 10 mM iodoacetic acid was added and the sample was incubated further at 37 °C. After glutathione peroxidase activity was more than 95% inhibited (10–15 min), 100 μL of 5 mM GSH was added and the sample was extensively dialyzed against water at 4 °C.

Aminoethylation of Glutathione Peroxidase. An aliquot of about 0.8 mg of phosphate-precipitated [^{75}Se]glutathione peroxidase was dissolved in 0.8 mL of water in an acid-hydrolysis tube. To reduce the enzyme, 0.05 mL of 20 mM GSH was added and the sample was incubated at 37 °C for 15 min, after which 0.05 mL of 20 mM dithioerythritol and 0.78 g of guanidinium hydrochloride were added. The tube was evacuated and incubated for 5 min at 100 °C, followed by incubation for 10 min at 37 °C. The sample was then cooled in ice, the vacuum was released, and 5 μL of ethylenimine was added. The tube was then reevacuated and incubated for 30 min at 37 °C. After the ethylenimine reaction, 10 μL of mercaptoethanol was added and the sample was incubated 30 min more at 37 °C to react with residual ethylenimine. After derivatization, the sample was dialyzed extensively against water at 4 °C.

Acid Hydrolysis of Derivatized Glutathione Peroxidase. The derivatized glutathione peroxidase samples were hydrolyzed in 6 N HCl under reduced pressure for 24 h at 110 °C. After hydrolysis, the solvent was removed under reduced pressure at room temperature. The acid-hydrolysis products from carboxymethylated glutathione peroxidase were taken up in 1.0 mL of 0.025 M sodium acetate buffer, pH 5.4. Purified ^{75}Se-labeled product from the acid hydrolysis of carboxymethylated [^{75}Se]glutathione peroxidase ([^{75}Se]product(Cm)) was obtained by DEAE-cellulose chromatography. The hydrolysis products of the carboxymethylated glutathione peroxidase were applied to a 1.5 × 10 cm DEAE-cellulose column equilibrated with 0.025 M sodium acetate, pH 5.4. The column was washed with 40 mL of the initial buffer and then an acetic acid gradient (0–0.4 M, 200 mL) was applied to elute the sample. The acid-hydrolysis products from aminoethylated [^{75}Se]glutathione peroxidase ([^{75}Se]products(Ae)) were taken up in 0.5 mL of water. [^{75}Se]Products(Ae) were not purified further before cochromatography experiments.

Column Chromatography. [^{75}Se]product(Cm) was applied together with an aliquot of Se-Cys([^{3}H]Cm) to three separate chromatography columns, and the collected fractions were assayed for ^{3}H and ^{75}Se to determine if there was coelution of these two compounds.

The anion-exchange gel used was a 0.9 × 15 cm column of DEAE-agarose (formate form) which had been washed with distilled water. After the sample was applied, the column was washed with 40 mL of water followed by a 100-mL linear gradient of 0.0 to 0.1 M formic acid.

The cation-exchange gel used was a 0.9 × 10 cm column of AG50W-X8 (H^+ form) equilibrated with 0.25 M citrate, 0.25 M phosphate, pH 2. After sample application, 30 mL of this

buffer was used as a wash, followed by a 100-mL linear gradient of the same buffer from pH 2 to 4.

The molecular exclusion gel used was a 1.5 × 90 cm column of Sephadex G-10 in 25 mM sodium acetate, pH 5.4. The flow rate was 0.15 mL/min.

Amino Acid Analysis. Automated amino acid analysis of the [^{75}Se]product(Cm) was performed in a 0.9 × 33 cm jacketed column of Durrum A6 resin. After application, the sample was eluted with Durrum Pico-Buffer System IV (a lithium citrate buffer system of which only the first two buffers were used in the region of interest) at 34 °C for 30 min and 60 °C thereafter.

Prior to amino acid analysis of the [^{75}Se]product(Ae), the sample was applied to a 0.9 × 15 cm column of AG50W-X8 equilibrated with 0.25 M NH_4Cl, pH 8.5, and then eluted with 0.25 M NH_4OH, pH 10. These steps were required to remove histidine, which would have eluted at a position close to, but not identical with, that of Se-Cys(Ae) on the amino acid analyzer.

Amino acid analyses of the [^{75}Se]product(Ae) and the [^{75}Se]products([^{14}C]Cm) were performed at 65 °C on a 0.9 × 33 cm jacketed column of Chroma-Beads Type B resin eluted with 0.05 M sodium citrate buffer, pH 5, with 0.1% Brij and 0.8 N in Na^+. Peak detection was by automated ninhydrin reaction.

Thin-Layer Chromatography. Thin-layer chromatography systems were standard systems used for separation of amino acids (Brenner and Niederwieser, 1967). Two-dimensional systems on silica gel G plates were: [system 1] first dimension, 1-butanol–acetic acid–H_2O (4:1:1); second dimension, phenol–H_2O (3:1, w/w); and [system 2] first dimension, chloroform–methanol–17% ammonia (2:2:1); second dimension, phenol–H_2O (3:1, w/w). A one-dimensional system on silica gel G plates was system 3, 1-propanol–28% ammonia (70:30). Two-dimensional thin-layer systems on cellulose MN 300 plates were: [system 4] first dimension, 1-butanol–acetone–diethylamine–H_2O (10:10:2:5); second dimension, 2-propanol–formic acid–H_2O (40:20:10); and [system 5] first dimension, 1-butanol–acetone–diethylamine–H_2O (10:10:2:5); second dimension, phenol–H_2O (3:1, w/w; gas phase equilibrated with 3% NH_3). ^{75}Se unknown (1200 cpm of [^{75}Se]product(Cm) or 1600 cpm of [^{75}Se]product(Ae)) and Se-Cys(Cm) or Se-Cys(Ae) standard (0.1 μmol) were cospotted on thin-layer plates. The plates were developed and sprayed with ninhydrin. Ninhydrin positive spots and the areas surrounding the spots were scraped from the plates and counted for ^{75}Se. When Se-Cys([^{3}H]Cm) (22 600 cpm) was used, the resins were taken up in 1 mL of water and transferred to scintillation vials containing 10 mL of Aquasol for ^{3}H counting.

Specific Carboxymethylation of the Glutathione Peroxidase Se. Derivatization of glutathione peroxidase with iodo[^{14}C]acetate specifically on the Se was done as follows. An aliquot of about 0.93 mg of phosphate-precipitated [^{75}Se]glutathione peroxidase was dissolved in 1.2 mL of 10 mM Tris buffer, 0.1 mM EDTA, pH 7.5. To reduce the enzyme, 40 μL of 0.1 M GSH was added and the sample was incubated at 37 °C for 8 min. After reduction, 50 μL of 0.1 M *N*-ethylmaleimide (in 95% ethanol) was added to react the protein thiol present in the mixture, and the sample was incubated at 37 °C for 25 min. In our experience *N*-ethylmaleimide has not been found to inhibit reduced rat liver glutathione peroxidase. A 50-μL aliquot of 0.03 M GSH was added to react with remaining *N*-ethylmaleimide and to reduce the glutathione peroxidase. The sample was incubated for 8 min at 37 °C, after which 0.1 mL of iodo[^{14}C]acetic acid (ca. 15 mM, 13.8 mCi/mmol) was added. After 15 min of incubation at 37 °C, 97% of the glutathione peroxidase activity was lost. Following the addition of 30 μL of 0.03 M GSH, the sample was dialyzed extensively against 0.5 M NaCl and then against water. After dialysis, this sample contained essentially all of the ^{75}Se. One milliliter of the dialyzed sample was hydrolyzed in 6 N HCl, and solvent was removed as described above. The residue was taken up in 1 mL of water and 0.5 mL was chromatographed on an amino acid analyzer column.

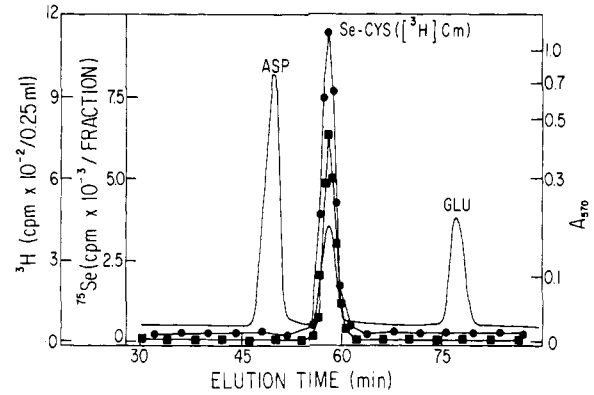

FIGURE 1: Amino acid analysis chromatogram of Se-Cys([^{3}H]Cm) and [^{75}Se]product(Cm). Asp and Glu (25 nmol of each) were added as internal standards. The $A_{570\ nm}$ (—) shows the ninhydrin profile from the amino acid analyzer. The eluate was collected in 1.0-mL fractions after ninhydrin reaction, and the fractions were assayed for ^{75}Se (■) from the [^{75}Se]-product(Cm) and ^{3}H (●) from the Se-Cys([^{3}H]Cm) standard.

Results

Glutathione Peroxidase Purification. After batch treatment with DEAE-cellulose, essentially all of the ^{75}Se was associated with glutathione peroxidase activity. Sodium dodecyl sulfate disc electrophoresis (Wu and Bruening, 1971) of the preparation showed a single ^{75}Se-containing peptide (R_f 0.89) that contained all of the ^{75}Se applied to the gel. The ^{75}Se band constituted 30–50% of the total protein on the gel, as determined by densitometry scans. On disc electrophoresis (Ornstein, 1964; Davis, 1964), ^{75}Se and glutathione peroxidase activity were coincident.

Cochromatography of [^{75}Se]Hydrolysis Products with Standards. [^{75}Se]product(Cm) was initially cochromatographed with Se-Cys([^{3}H]Cm) on a DEAE-agarose anion-exchange column, on an AG50W-X8 cation-exchange column, and on a Sephadex G-10 gel permeation column. With all three columns, coelution of ^{3}H and ^{75}Se was observed.

[^{75}Se]product(Cm) and the standard of Se-Cys([^{3}H]Cm) applied to an amino acid analyzer coeluted at 58 min, as shown in Figure 1. Some of the aspartate peak appears to be a contaminant from the applied sample.

On all thin-layer systems [^{75}Se]product(Cm) and [^{75}Se]-product(Ae) showed cochromatography with Se-Cys(Cm) and Se-Cys(Ae), respectively. Table I shows the results of thin-layer chromatography. In a typical experiment, 59–78% of the ^{75}Se and ^{3}H was recovered in a carrier spot, and no significant amounts of ^{75}Se or ^{3}H were recovered in the areas surrounding the spots. All plates with the [^{75}Se]product(Cm) showed a single purple spot after reaction with ninhydrin. Plates of [^{75}Se]products(Ae) showed an intense Se-Cys(Ae) carrier spot and fainter spots from other amino acids.

Amino acid analysis of standard Se-Cys(Ae) and [^{75}Se]-product(Ae) showed coelution (Figure 2). The ammonia was a contaminant from the sample. The ninhydrin peak at 67.7 min is aminoethylcysteine. The large peak at 75.9 min is the coelution of Se-Cys(Ae) standard and [^{75}Se]product(Ae).

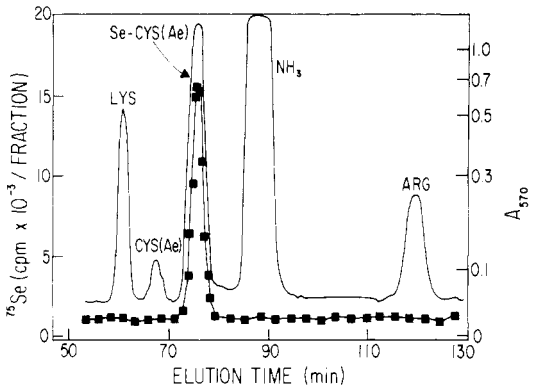

FIGURE 2: Amino acid analyzer chromatogram of Se-Cys(Ae) and [^{75}Se]product(Ae). Lys and Arg (25 nmol of each) were added as internal standards. The $A_{570\ nm}$ (—) shows the ninhydrin profile from the amino acid analyzer. The eluate was collected in 0.8-mL fractions after ninhydrin reaction, and the fractions were assayed for ^{75}Se (■).

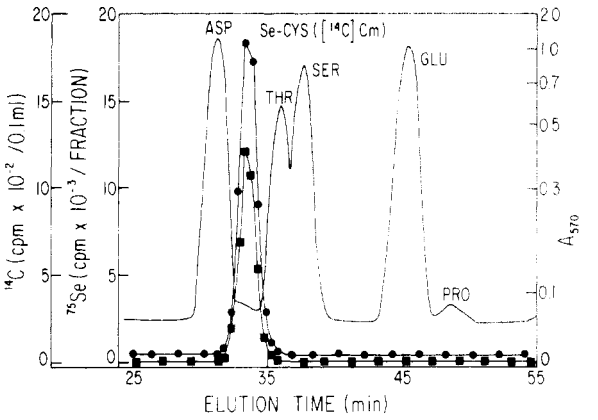

FIGURE 3: Amino acid analyzer chromatogram of [^{75}Se]product([^{14}C]-Cm). The $A_{570\ nm}$ (—) shows the ninhydrin profile from the amino acid analyzer. The eluate was collected in 0.97-mL fractions after ninhydrin reaction, and the fractions were assayed for ^{75}Se (■) and ^{14}C (●).

TABLE I: Cochromatography of ^{75}Se-Labeled Products and Standards on Thin-Layer Plates.

Components of sample applied	TLC system[a]	1st dimension R_f	2nd dimension R_f	^{75}Se recovery (%)	^{3}H recovery (%)
[^{75}Se]Product(Cm) + Se-Cys([^{3}H]Cm)[b]	1	0.34	0.33	61	66
[^{75}Se]Product(Cm) + Se-Cys([^{3}H]Cm)	2	0.56	0.34	73	78
[^{75}Se]Product(Cm) + Se-Cys(Cm)	4	0.25	0.38	78	
[^{75}Se]Product(Ae) + Se-Cys(Ae)	1	0.07	0.17	69	
[^{75}Se]Product(Ae) + Se-Cys(Ae)	2	0.78	0.09	76	
[^{75}Se]Product(Ae) + Se-Cys(Ae)	5	0.49	0.86	59	
[^{75}Se]Product(Ae) + Se-Cys(Ae)	3	0.45		75	

[a] See text under Experimental Procedure for description of thin layer systems. [b] Plate was developed twice in the first dimension.

[^{75}Se]Product(Ae) also cochromatographed with Se-Cys(Ae) on CM-cellulose (applied and eluted in 5 mM sodium acetate, pH 5.4). Of the ^{75}Se applied, 86% was recovered in the Se-Cys(Ae) peak, which eluted well after the void volume.

Specific Labeling of Glutathione Peroxidase. The glutathione peroxidase specifically derivatized with iodo[^{14}C]acetic acid was assayed for ^{75}Se and ^{14}C after dialysis. From the specific activities of the two isotopes, it was determined that 0.92 and 0.88 iodoacetate were bound for every Se atom in the intact protein and the acid-hydrolyzed sample, respectively.

Figure 3 shows the amino acid analyzer chromatogram of the [^{75}Se]products([^{14}C]Cm). The ^{75}Se and ^{14}C cochromatographed in the major peak. Ninety-three percent of the ^{14}C was recovered in the major ^{14}C–^{75}Se peak, and 90% of the ^{75}Se applied to the column was recovered in that peak. The ratio of iodoacetates per Se atom in the peak was 0.93:1.0. No ^{14}C radioactivity or ninhydrin absorbance was found in the chromatogram in the region expected for carboxymethylcysteine at an elution time of 28 min.

Discussion

In general, the partial purification of glutathione peroxidase by the described procedure is very reproducible, gives good recoveries of enzyme activity, and is expedient. One batch of enzyme may be prepared in 4 days. As shown by electrophoresis, essentially all of the Se in the preparation is the glutathione peroxidase. In their purification of glutathione peroxidase, Nakamura et al. (1974) employed basically similar tactics, and they obtained purified glutathione peroxidase after repeated DEAE-Sephadex chromatography. With these extra steps, they experienced greater losses of enzyme. For the purpose of identification of the Se moiety, partially purified glutathione peroxidase in good yields was used to advantage in our experiments. Since glutathione peroxidase can be used to assay hydroperoxides (Heath and Tappel, 1976), this expedient method for fractionation of this enzyme should be of utility in other investigations.

The preferred method of prosthetic group structural determination would be to isolate sufficient amounts of pure substance so that definitive structural analyses could be performed. However, it is difficult to purify rat liver glutathione peroxidase in amounts sufficient for chemical characterization of the Se moiety, and the Se compound cannot be isolated as a native prosthetic group but only as stable derivatives. Even these Se derivatives are unstable to techniques such as mass spectrometry, wherein we found that extensive decomposition occurs. The next best method of structural identification is chromatographic comparison with known standards; in this case, Se-Cys(Cm) and Se-Cys(Ae). Both standards were prepared by known synthetic routes, and both behaved on all chromatographic systems as predicted by theory. Se-Cys(Cm) was further characterized by elemental analysis to have the same elemental composition as predicted by theory. Further derivatization and mass spectrometry of both Se-Cys(Ae) and Se-Cys(Cm) also were consistent with theoretical expectations.

From the consistent cochromatography of the two standard Se compounds with the respective ^{75}Se hydrolysis products in so many different systems, it is reasonably certain that the isolated [^{75}Se]product(Cm) and [^{75}Se]product(Ae) were Se-Cys(Cm) and Se-Cys(Ae), respectively, and that the form of Se in glutathione peroxidase is Se-Cys. Similar methods of cochromatography with known Se compounds also enabled Cone et al. (1976) to identify Se-Cys as the Se moiety of the glycine reductase of *Clostridium sticklandii*.

Recovery of the Se from the derivatized and hydrolyzed enzyme typically was in the 60–90% range, indicating that all of the Se atoms in glutathione peroxidase are Se-Cys and that the Se is not in two or more forms in the enzyme.

The nature of Se compounds from mammalian tissues remains controversial (Ganther, 1974; Burk, 1976). It has been shown that animals may utilize Se-Met in partial place of methionine in protein synthesis (Ochoa-Solano and Gitler, 1968; Cary et al., 1973). McConnell and Wabnitz (1957) reported finding selenocystine in dog liver hydrolysates, but Cummins and Martin (1967) and Jenkins (1968) could find no evidence of seleno amino acids in animal tissue. Schwarz and Foltz (1958) also concluded that α-factor 3 (an acid-hydrolysis product from hog kidney powder) was not selenocystine, selenomethionine, or selenite based on the relative abilities of these compounds to prevent liver necrosis in rats. However, Cone et al. (1976) have commented on the need to protect Se-Cys prior to hydrolysis and isolation from glycine reductase. Since Se-Cys is labile to acid hydrolysis (Huber and Criddle, 1967) and general isolation procedures (Cone et al., 1976), it is not surprising that previous studies have not found more Se-Cys from glutathione peroxidase of animal tissue. Recently, Olson and Palmer (1976) identified compounds containing [^{75}Se]Se-Cys in Pronase digests of liver and kidney acetone powders from rats administered [^{75}Se]selenite. Their work demonstrates the necessity of utilizing mild procedures for hydrolysis of proteins that contain unprotected Se-Cys. The ^{75}Se-Cys they isolated probably came from glutathione peroxidase.

Diplock et al. (1971) have shown that roughly 60% of the selenium in rat liver supernatant is non-zinc–hydrochloric acid reducible. This finding has been interpreted to mean that selenium is organically bound (Rhead et al., 1974). Stults et al. (1977) have shown that glutathione peroxidase contains at least 40% of the ^{75}Se in rat liver supernatant 3 days after [^{75}Se]selenite injection, which correlates well with the findings of Diplock et al. (1971).

A few published reports have focused on the nature of the Se moiety in glutathione peroxidase. Oh et al. (1974) have reported loss of Se from ovine erythrocyte glutathione peroxidase after alkaline or acid treatment. The product has not yet been identified, although the authors indicated the compound was not selenite or selenate. Prohaska et al. (1977) have shown that cyanide will release Se from glutathione peroxidase. They speculated that the Se in oxidized glutathione peroxidase was in a form related to a selenenic acid. Fletcher (1975) and Hsu (1976) have shown release of ^{75}Se from [^{75}Se]glutathione peroxidase by sodium borohydride, but the product has not been positively identified. Because these studies were done on enzyme with the selenium unprotected, the selenium recovered from the enzyme was probably in decomposition products from the Se-Cys.

Flohé et al. (1976) found that none of the tryptic peptides of glutathione peroxidase contained appreciable amounts of Se. He speculated the Se was lost after extensive iodoacetamide derivatization of the enzyme. We also have found that if the carboxymethylated selenium glutathione peroxidase is incubated with 6 N guanidinium hydrochloride and 100 mM iodoacetate the selenium is released and has the characteristics of diacetylselenium. This artifact probably arises from formation and decomposition of a trialkylselenium intermediate, which is a common reaction of seleno ethers and alkyl halides (Irgolic and Kudchadker, 1974).

The metabolic pathways of selenium compounds in animals have been the subject of intense research for a number of years. A variety of organoselenium compounds prevent dietary liver necrosis in rats (Schwarz and Fredga, 1969; Schwarz and Foltz, 1958) and exudative diathesis and pancreatic fibrosis in chicks (Cantor et al., 1975a,b). If the effect of selenium compounds in selenium-deficiency diseases is via glutathione peroxidase, there must be a pathway of Se from various precursors into the Se-Cys of the enzyme.

If Se-Cys is biosynthesized prior to glutathione peroxidase synthesis in vivo, it could be made from Se-Met via selenocystathionine. Schwarz and Foltz (1958) have shown that selenocystathionine is as effective in prevention of liver necrosis as Se-Met, Se-Cys, and selenite. Synthesis of Se-Cys from selenite in vivo may occur via a cysteine synthase (EC 4.2.1.22) like reaction (Olson and Palmer, 1976). Cysteine synthase activity has been demonstrated in rat liver preparations using Na_2S and serine as substrates (Huovinen, 1968), and Hsieh and Ganther (1975) have shown H_2Se formation in systems containing GSH and glutathione reductase. To form Se-Cys, H_2Se would act similarly to Na_2S in the reaction.

The presence of a Se-Cys moiety specifically attached to a protein poses the question of the mechanism by which this attachment is made. There has been no evidence for the existence of genetic codons, transfer RNAs, or charging enzymes for Se-Cys, and it is highly unlikely that Se-Cys is incorporated specifically via normal transcription and translation. The Se-Cys in glutathione peroxidase, therefore, probably is formed posttranslationally either by attachment of the presynthesized Se-Cys molecule onto the protein backbone via linkage to a side chain or by modification of the side chain of an incorporated amino acid such as Cys or Ser to Se-Cys.

Flohé and Günzler (1974) have shown that only reduced glutathione peroxidase is inhibited by iodoacetate. In our specific labeling experiment, it was shown that the only iodoacetate-reactive site associated with enzymatic activity is the Se moiety in the intact enzyme. Therefore, the Se-Cys is the catalytic moiety of the enzyme, and the Se-Cys selenol (–SeH) is the reduced form of the catalytic site. Using X-ray photoelectron spectroscopy, Chiu et al. (1977) and Wendel et al. (1975) found evidence consistent with a selenol structure for the selenium in glutathione peroxidase. Ganther et al. (1976) proposed a possible mechanism for the glutathione peroxidase reaction in which a glutathione peroxidase selenol would be oxidized by the peroxide substrate to a selenenic acid. The selenenic acid is reduced to the selenol by sequential reactions with two GSH molecules to complete the enzymatic cycle. This mechanism is consistent with our finding that only one iodoacetate is bound per selenium in the reduced state. Some evidence that the oxidized form of the selenium may be a selenenic acid has been provided by X-ray photoelectron spectroscopy (Wendel et al., 1975) and cyanide inhibition of oxidized enzyme (Prohaska et al., 1977). It is unlikely that a selenosulfide (–Se–S–) is in the oxidized enzyme. It also is unlikely that a selenol and a thiol are in the reduced form of the enzyme, as the reduced enzyme should react with two iodoacetates per Se, whereas it only reacts with one iodoacetate per Se. The work of Flohé et al. (1971) also can be interpreted in this manner. Another possibility is that the oxidized form of the enzyme contains a diselenide (–Se–Se–), which also is consistent with the results of the labeling experiment.

These problems and questions provide intriguing avenues of research along which we are progressing.

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