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Identification of Selenocysteine in Glutathione Peroxidase by Mass Spectroscopy†

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ABSTRACT: A convenient procedure was developed for identifying selenocysteine in selenoproteins by mass spectroscopy, based on formation of the 2,4-dinitrophenyl (DNP) derivative. Pure ovine erythrocyte glutathione peroxidase was reduced with sodium borohydride and reacted with 1-fluoro-2,4-dinitrobenzene at neutral pH under anaerobic conditions in 4 M guanidine. The inactivated enzyme was hydrolyzed with 6 N HCl for 20 h at 110 °C under anaerobic conditions. Following extraction of the hydrolysate with benzene, *Se*-(2,4-dinitrophenyl)selenocysteine in the aqueous phase was separated from non-DNP-amino acids by gel-filtration chromatography and then separated from other water-soluble DNP-amino acids by reversed-phase high-performance liquid chromatography. The *Se*-(2,4-dinitrophenyl)selenocysteine was converted to *Se*-methyl-*N*-(2,4-dinitrophenyl)selenocysteine by the addition of sodium barbital to induce an intramolecular Se → N shift (Smiles rearrangement) under anaerobic conditions, in the presence of methyl iodide to trap the liberated selenol group. Following esterification of the product's carboxyl group with methanol and hydrochloric acid, it was subjected to direct probe mass spectroscopy and identified as the methyl ester of *Se*-methyl-*N*-(2,4-dinitrophenyl)selenocysteine. This procedure allows selenocysteine to be isolated quite easily as a readily identifiable derivative and has permitted the first identification of a seleno amino acid in a protein by mass spectroscopy.

After the recognition that erythrocyte glutathione peroxidase (EC 1.11.1.9) (GSH peroxidase) was a selenium-containing enzyme (Rotruck et al., 1973; Flohe et al., 1973; Oh et al., 1974), attempts were made to identify the chemical form of the selenium moiety. Some initial attempts to identify low molecular weight forms of selenium released from the enzyme by various procedures were unsuccessful (Oh et al., 1974; Chiu et al., 1975). No seleno amino acids were detected following proteolytic digestion of GSH peroxidase that had been reduced and treated with iodoacetamide (Flohe et al., 1976). Selenocysteine was first identified in a microbial selenoprotein of the glycine reductase complex, after reduction and alkylation of the selenium to form *Se*-carboxylmethyl or *Se*-aminoethyl derivatives (Cone et al., 1976). Forstrom et al. (1978) used similar methods to derivatize the selenium in rat liver GSH peroxidase and showed that it cochromatographed with the carboxymethyl and aminoethyl derivatives of a selenocysteine standard. They were unable to obtain the mass spectrum of any selenium-containing compound from the enzyme, although they obtained mass spectra of *N*-acetyl-*O*-methyl derivatives of the Se-alkylated selenocysteine standards. Cone et al. (1976) experienced similar difficulties in their attempts to obtain mass spectra and noted the susceptibility of the selenoether derivatives of selenocysteine to oxidation. To date, cochromatography has been the primary method for identifying seleno amino acids, and no one has identified the selenium moiety of any known selenoprotein by mass spectroscopy.

We now report a new experimental approach for identification of selenocysteine in GSH peroxidase by mass spectroscopy, based on derivatization of the reduced enzyme with Sanger's reagent (FDNB)[1] to form *Se*-(2,4-dinitrophenyl)-selenocysteine. The presence of the DNP moiety made the seleno amino acid relatively easy to isolate from acid hydrolysates and permitted convenient detection during chromatography. The *Se*-DNP-selenocysteine from the enzyme was identified by mass spectroscopy following transformation to the *Se*-methyl-*N*-DNP-selenocysteine methyl ester.

Experimental Procedures

Materials. Selenocystine, FDNB, N^{ϵ}-DNP-lysine, $O^{4'}$-DNP-tyrosine, *N*-DNP-arginine, and guanidine hydrochloride (Gdn·HCl) were purchased from Sigma. N^{im}-DNP-histidine was a gift from Dr. Vincent Massey. *Se*-Methylselenocysteine was obtained from Cyclo Chemical. Nitrogen (Matheson, prepurified grade) was passed through an Oxyclear trap (Pierce Chemical). All solvents used for HPLC and mass spectroscopy were redistilled.

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[1] Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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Model Compounds. *Se*-DNP-selenocysteine was prepared by reduction of selenocystine with sodium borohydride and reaction with FDNB at pH 6 under anaerobic conditions. *S*-DNP-cysteine was prepared from cystine in the same way at pH 7. *Se*-Methyl-*N*-DNP-selenocysteine was prepared by reacting *Se*-methylselenocysteine with FDNB in a solution of bicarbonate (Kesner et al., 1967). Alternatively, *Se*-methyl-*N*-DNP-selenocysteine was prepared from *Se*-DNP-selenocysteine by using the Smiles rearrangement in the presence of methyl iodide (see below). *N*-Acetyl-*N*-DNP-lysine was prepared by treating N^{ϵ}-DNP-lysine at 25 °C with acetic anhydride–methanol (1:4) for 48 h. Methyl esters of the DNP-amino acids were formed by overnight reaction with 0.1 N HCl in methanol at 25 °C, followed by refluxing at 70 °C for 2 h. The DNP derivatives were purified by TLC. The structures of the model selenium and sulfur compounds were confirmed by proton NMR.[2]

Chromatography. Thin-layer chromatography was done on precoated silica gel plates (Brinkmann) in benzene–pyridine (80:20 v/v). DNP compounds were located by ultraviolet light at 350 nm, scraped from the plate, and eluted with solvent. The methanol–water solvent systems used for HPLC were degassed by evacuation with a water aspirator and then filtered through a Metricel GN-6 filter, pore size 0.45 μm (Gelman). Two HPLC columns were used: (1) a semipreparative (10 × 250 mm) reversed-phase Whatman Partisil M9 ODS column, monitored at 254 nm with an ISCO UA-2 analyzer, and (2) an analytical (4 × 250 mm) Bio-Rad reversed-phase Bio-Sil ODS-5S column, monitored at 254 nm with a Waters differential UV detector. Aliquots (up to 20 μL) of column fractions were analyzed directly for Se at 196 nm by graphite-furnace atomic absorption spectrometry (Hitachi Model 170-70) with Zeeman-effect background correction under the following conditions: drying, 20 A (100 °C), 25 s; ashing, 120 A (1450 °C), 30 s; atomization, ramp (60 A/s) to 300 A (2800 °C), 10 s. Pooled column fractions and other samples were analyzed for selenium after wet ashing by fluorometric analysis (Oh et al., 1974).

Derivatization of GSH Peroxidase. Highly purified ovine erythrocyte enzyme having a specific activity of 1560 units/mg of protein (460 units/μg of Se) was assayed and isolated as previously described (Kraus et al., 1980) and stored at 4 °C in 10% ethanol–50 mM potassium phosphate, pH 7.2. At the time of derivatization, the specific activity was 1400 units/mg, and the enzyme was in the oxidized form (form C), as shown by inhibition with cyanide (Kraus et al., 1980). Sixty milliliters of enzyme solution (24 mg of protein, 79.8 μg of Se), cooled to 2 °C, and 60 mL of 8 M Gdn·HCl were purged with nitrogen for 1 h. The enzyme was then reduced by adding 6 mg of $NaBH_4$. Testing of an aliquot with iodoacetate (Kraus et al., 1980) showed that the activity was inhibited 97%. After the reduced enzyme was transferred to a 250-mL flask closed with a rubber septum, the guanidine solution was added, then 20 mL of 0.11 M FDNB in absolute ethanol. A combination pH electrode was introduced through a narrow glass tubing sleeve in the septum and the pH carefully adjusted to 7.5–8.0 with 1 N KOH under a nitrogen purge. The mixture was allowed to react for 2 h at 25 °C in the dark under nitrogen purge and then sealed and left overnight. The mixture was acidified with 5 drops of 12 N HCl and exhaustively dialyzed against 20% aqueous ethanol (ethanol–water, 20:80 v/v) at 25 °C in the dark. The yellow precipitated protein was taken to dryness by rotary evaporation at 37 °C and then suspended in methanol and divided into three portions for hydrolysis.

Acid Hydrolysis. The FDNB-treated GSH peroxidase samples were evaporated to dryness at 37 °C by means of a mechanical vacuum pump in 19 × 150 mm Teflon-stoppered vacuum hydrolysis tubes (Pierce), and 4 mL of N_2-purged 6 N HCl was added to each tube. After the solutions were purged with N_2 for 1 h, the tubes were evacuated, closed, and heated in a block at 110 °C for 20 h in the dark. The acid hydrolysates were extracted with benzene, and the water-soluble DNP-amino acids in the aqueous phase were evaporated to dryness at 37 °C. The recovery of Se in the aqueous phase was 100% of that hydrolyzed (80 μg). The hydrolysate, dissolved in 2 mL of 20% ethanol, was chromatographed on a Sephadex G-10 column (see Results).

Smiles Rearrangement. Following purification through the Bio-Rad HPLC column (see Results), the selenium from GSH peroxidase or standard *Se*-DNP-selenocysteine (about 10 μg for each) was dissolved in 0.5 mL of methanol and purged with nitrogen for 15 min. After the addition of 0.05 mL (0.8 mmol) of methyl iodide, 0.2 mL of 0.1 M sodium barbital in methanol was added and the mixture kept under nitrogen for 75 min at 25 °C and then evaporated to dryness. The product was dissolved in 1 mL of 0.18 N HCl in methanol and allowed to stand overnight at 25 °C and then refluxed for 2 h at 70 °C. The sample was applied to a silica gel thin-layer plate and chromatographed in benzene–pyridine (80:20 v/v). The UV-absorbing band at R_f 0.5 was scraped up and the compound eluted with benzene–pyridine (1:1 v/v). After concentration of the sample with a stream of nitrogen at 25 °C, a 10–15-μL aliquot containing about 2 μg of compound was transferred to a capillary tube by means of a Hamilton syringe and allowed to dry overnight and then subjected to mass spectroscopy.

Mass Spectra. Mass spectra were obtained by direct probe analysis at 70-eV ionization energy with 8-keV acceleration on a Kratos MS-9 instrument equipped with a DS-50 data system.[3] The probe temperature was 165 °C.

Results

Chromatography. The water-soluble, benzene-extracted acid hydrolyzate of FDNB-treated GSH peroxidase (80 μg of Se) was applied to a Sephadex G-10 column and eluted with 20% aqueous ethanol to separate DNP-amino acids from the other free amino acids (Figure 1). Fractions 1–30 were colorless and contained a minor part of the selenium. Fractions 31–65 were bright yellow and contained 80% (64 μg) of the selenium applied to the column, as estimated by atomic absorption. The pooled DNP-amino acids were dried by rotary evaporation at 37 °C and then taken up in 1 mL of 20% aqueous methanol and chromatographed on the semipreparative reversed-phase HPLC column in three portions (Figure 2). The fractions were pooled into four peaks and then dried by rotary evaporation and stored at 4 °C. All of the selenium eluted from the column was in peak III, which coincided with the elution position of authentic *Se*-DNP-selenocysteine, as well as *N*-DNP-arginine. Fractions 21–32 were pooled (38.9 μg of Se). On the basis of the absorbance at 254 nm of *Se*-DNP-selenocysteine, all of the absorbance in peak III was accounted for by the selenium content. Therefore, the presence of *N*-DNP-arginine is excluded. Peak IV was identified as N^{ϵ}-DNP-lysine, on the basis of cochromatography with the known compound and mass spectrscopy (see below). *S*-

[2] The NMR measurements were obtained through the courtesy of Dr. H. Reich of the Department of Chemistry.

[3] The mass spectra were obtained in cooperation with Dr. H. Schnoes of the Deparment of Biochemistry.

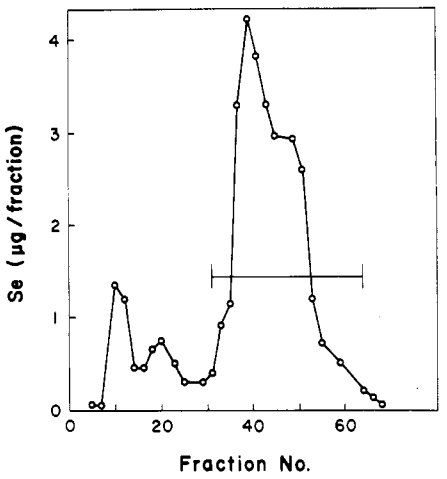


FIGURE 1: Separation of DNP-amino acids from other amino acids by gel-filtration chromatography. A 2-mL aliquot of acid-hydrolyzed FDNB-treated GSH peroxidase was applied to a 2.5 × 12 cm column of Sephadex G-10 and eluted with 20% aqueous ethanol at 4 °C, 30 mL/h. Fractions of 5 mL were collected and analyzed for Se by atomic absorption.

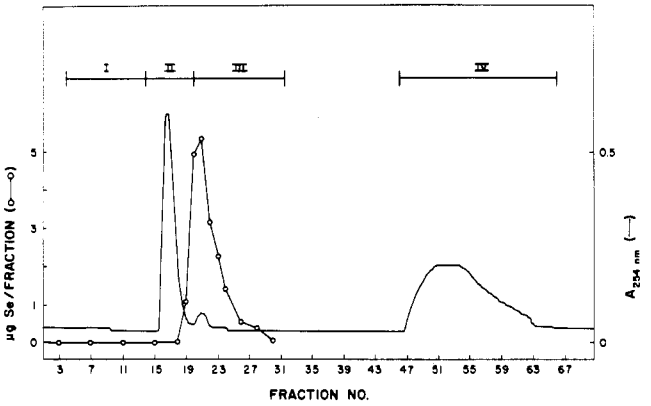


FIGURE 2: Separation of DNP-amino acids on reversed-phase HPLC column. Aliquots (0.33 mL) of the pooled fractions (31–65) from the Sephadex G-10 column (Figure 1) were applied to a 10 × 250 mm semipreparative Whatman M9 Partisil ODS column, particle size 10 μm, and eluted at 25 °C with methanol–water (20:80 v/v) at 2.9 mL/min. Fractions of 2.8 mL were collected and analyzed for Se by atomic absorption.

DNP-cysteine was shown to be eluted in the position of peak I. Peak II was not identified but may consist in part of N^{im}-DNP-histidine (see below). GSH peroxidase from bovine erythrocytes is known to contain a histidine residue near the active site (Ladenstein et al., 1979). *O*-DNP-tyrosine was eluted after peak IV. Part of the selenium (12 μg) from peak III was further purified in two portions on an analytical Bio-Rad reversed-phase column (Figure 3). There were two major UV-absorbing peaks, centered on fractions 17 and 21, respectively. Selenium was eluted mainly with the second peak. The elution position of authentic *Se*-DNP-selenocysteine corresponded to that of the second peak. Fractions 20–29 for both the unknown and the standard were pooled, and each sample was dried by rotary evaporation and then carried through the Smiles rearrangement and esterification procedure prior to mass spectroscopy (see Experimental Procedures).

A sample of N^{im}-DNP-histidine was subjected to HPLC on both columns to determine its elution position. On the semipreparative reversed-phase column, it was eluted in the same position as peak II of the enzyme sample (Figure 2), and the two samples could not be resolved when mixed. On the Bio-Rad column, the N^{im}-DNP-histidine was eluted just before *Se*-DNP-selenocysteine, in the same position as the first major

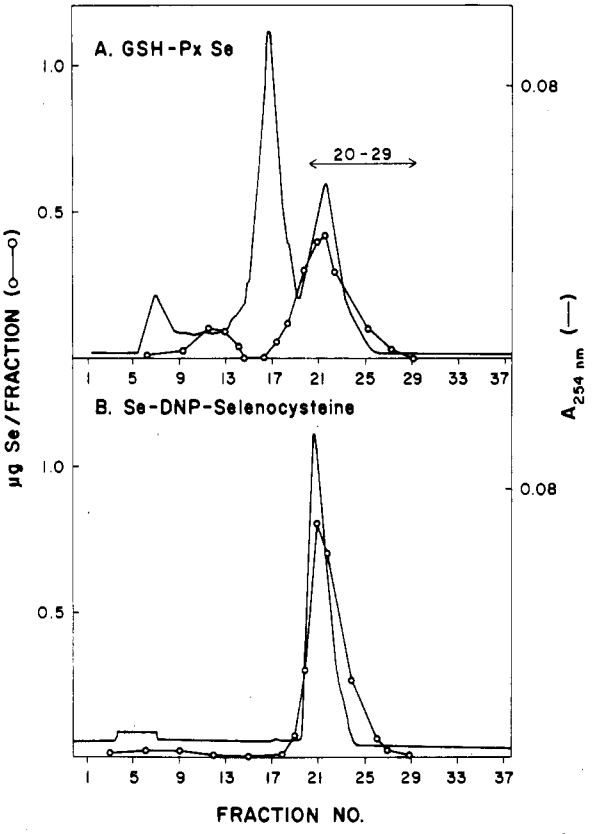


FIGURE 3: High-resolution reversed-phase HPLC purification of *Se*-DNP-selenocysteine. (A) Aliquots (6 μg of Se) of pooled fractions of peak III from the semipreparative HPLC column (Figure 2) were applied to a 4 × 250 mm Bio-Rad reversed-phase Bio-Sil ODS-5S column and eluted with methanol–water (30:70 v/v) at 25 °C, 0.8 mL/min. Fractions of 0.80 mL were collected and analyzed for Se by atomic absorption. (B) Standard *Se*-DNP-selenocysteine (6 μg of Se).

UV peak (Figure 3A, fraction 17) in the GSH peroxidase hydrolysate. On the basis of cochromatography, it is tentatively concluded that the unidentified peak eluted just before *Se*-DNP-selenocysteine contains N^{im}-DNP-histidine.

Identification of Se-DNP-selenocysteine by Mass Spectroscopy. The mass spectrum (Figure 4A) of the selenium isolated from GSH peroxidase and subjected to the Smiles rearrangement was identical with that of the methyl ester of authentic *Se*-methyl-*N*-DNP-selenocysteine (Figure 4B) prepared from selenocysteine; the mass spectrum of the standard prepared from *Se*-methylselenocysteine also had the same spectrum (not shown). The molecular ion was a prominent peak at m/e = 363 and showed the typical isotope pattern for selenium. Other selenium peaks were observed at m/e = 317 (loss of NO_2), 304 (loss of CO_2CH_3), and 109 (the CH_3SeCH_2 fragment). The m/e = 254 fragment corresponds to the loss of CH_3–Se–CH_2.

Identification of N^{ϵ}-DNP-lysine. Peak IV (Figure 2) off the Whatman reversed-phase column was suspected to be the lysine derivative, on the basis of coelution with the standard compound. The *N*-acetyl-*O*-methyl ester derivatives of the unknown and *N*-DNP-lysine were prepared as described (see Experimental Procedures) and were shown to be identical by mass spectroscopy. The molecular ion was present at m/e = 368; fragments included m/e = 352 (loss of O), 337 (loss of OCH_3), 309 (loss of CO_2CH_3), 250 (loss of CO_2CH_3 and $NHCOCH_3$), and 196 (loss of $-(CH_2)_3CH(NHCOCH_3)-CO_2CH_3$). From spectrophotometric measurements of peak IV with the extinction coefficient determined for *N*-DNP-lysine, a value of 4.3 lysine residues per subunit of ovine

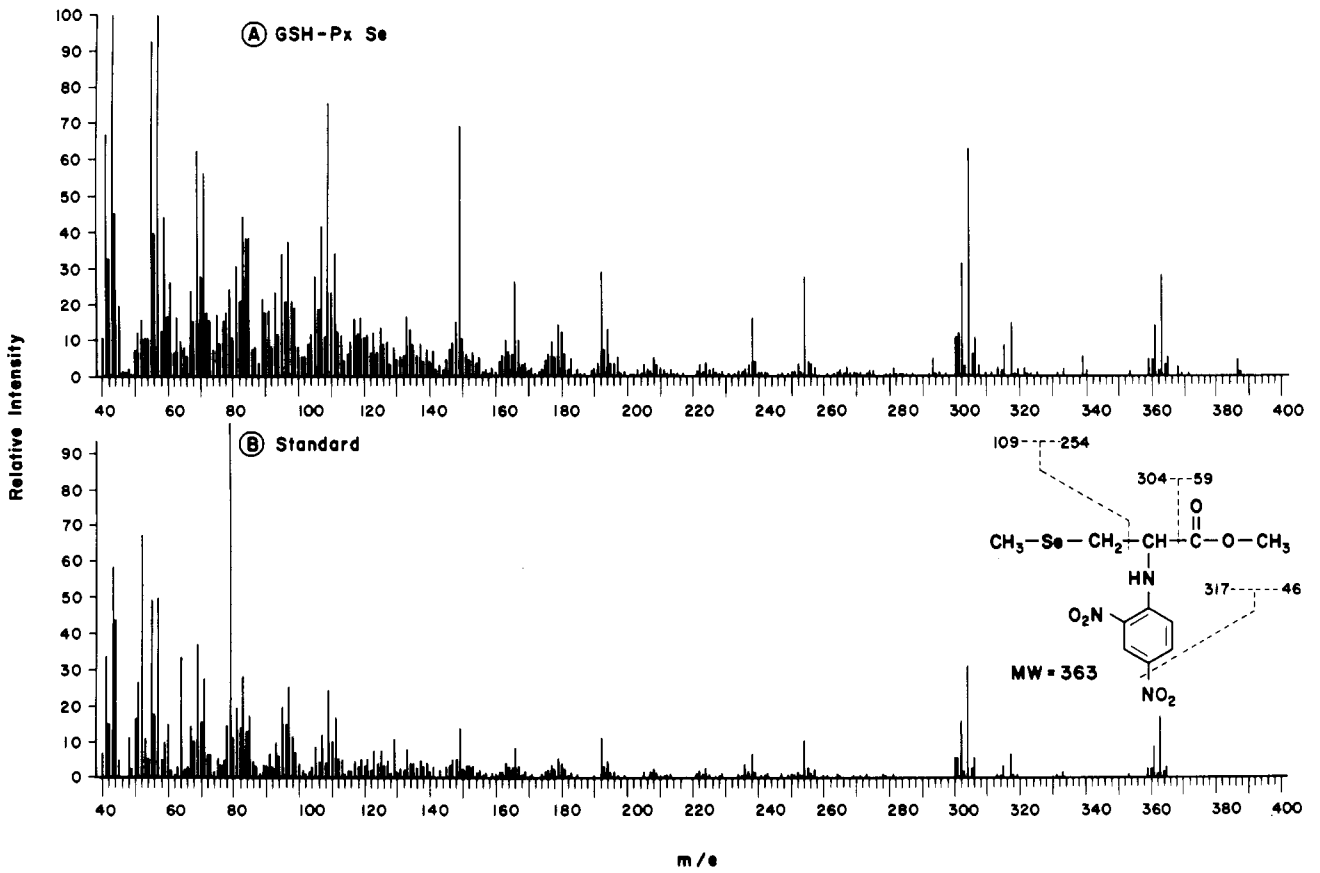


FIGURE 4: Mass spectrum of methyl ester of *Se*-methyl-*N*-DNP-selenocysteine. (A) Selenium isolated from GSH peroxidase. (B) Standard prepared from *Se*-DNP-selenocysteine. The large peak at $m/e = 79$ in sample B is caused by incomplete removal of pyridine used in eluting the compound after purification by TLC.

erythrocyte GSH peroxidase was calculated, compared to estimated values of 5–7 residues reported for the bovine erythrocyte enzyme [see Ladenstein et al. (1981)].

Identification of N-Terminal Amino Acid. A portion of the benzene extract of the acid hydrolysate of FDNB-treated GSH peroxidase was subjected to preparative TLC on silica gel in $CHCl_3$–methanol–acetic acid (90:8:3), giving four yellow bands. When these were eluted and subjected to thin-layer electrophoresis on cellulose at pH 5.3, three remained at the origin, and one moved toward the anode with the same mobility as *N*-DNP-alanine. There was no sign of a dicarboxylic DNP-amino acid. The acidic compound was esterified in 1 mL of methanol–0.1 N HCl and chromatographed on a silica gel plate in benzene–pyridine (80:20). The unknown compound had an R_f of 0.43, identical with that of the methyl ester of *N*-DNP-alanine. After elution with chloroform, both compounds were subjected to direct probe mass spectroscopy. The mass spectra were identical; the molecular ion was observed at $m/e = 269$ plus fragments including $m/e = 210$ (loss of $-CO_2CH_3$), 194 (loss of $-CO_2CH_3$ and O), 164 (loss of $-CO_2CH_3$ and $-NO_2$), and 149 (loss of $-CO_2CH_3$, $-NO_2$, and $-CH_3$). It is concluded that the N-terminal amino acid of ovine erythrocyte GSH peroxidase is alanine.

Discussion

Sanger's reagent (FDNB) has been widely used for decades to make derivatives of amino acids that are useful in analysis. Our work shows that selenocysteine can be included in the list of amino acids in proteins whose side chains react with FDNB, along with lysine, histidine, tyrosine, and cysteine (Sanger, 1945; Swenson et al., 1982). More importantly, such derivatization leads to a very convenient method for isolating selenocysteine as its DNP derivative and has permitted the first identification of the form of selenium in a selenoprotein by mass spectroscopy.

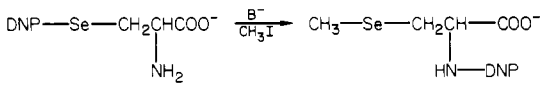
Previous investigators used conventional aliphatic agents such as iodoacetic acid to derivatize the selenium in various selenoproteins. Our starting point was the idea that if selenium could be coupled to an aromatic ring, the resulting derivative would be more stable and have desirable solubility properties. Knowing that *o*-nitrobenzene derivatives of selenium compounds (even selenenic acids) are quite stable (Twiss, 1914; Reich et al., 1982) and that selenols react with FDNB very easily, we reacted the selenium of reduced GSH peroxidase with FDNB. This proved to have several technical advantages. First, DNP-amino acids are readily detected, both visually and by ultraviolet light, by means of their strong absorption bands at approximately 330–360 and 254 nm. Second, they are easily fractionated. Simple N-terminal amino acids form DNP derivatives that are extractable into benzene from acid solutions. DNP derivatives of amino acid residues whose side chains react with FDNB remain in the aqueous phase and are resolved by reversed-phase HPLC in isocratic methanol–water systems. Even though selenium compounds are often difficult to separate from their sulfur analogues by TLC or other chromatographic systems, *Se*-DNP-selenocysteine and *S*-DNP-cysteine were easily separated in an isocratic HPLC system.

Pure *Se*-DNP-selenocysteine was obtained from the acid hydrolysate of GSH peroxidase after three chromatographic steps; the isolation procedure could probably be simplified further, perhaps to a single HPLC column, by appropriate choice of conditions or use of a solvent gradient. Reed et al. (1980) developed a sensitive HPLC method for various thiols

on the basis of S-carboxymethylation followed by reaction with FDNB to obtain the *N*-DNP derivatives, which were separated on a single column with a gradient system. The sensitivity of our method could also be enhanced by taking fuller advantage of the ultraviolet absorption of the DNP group (Reed et al., 1980) or by using radioactive FDNB (Swenson et al., 1982). In the present work, a relatively large amount of enzyme (24 mg) was used, but the final aliquot taken through the rearrangement–esterification procedure for mass spectroscopy contained only 10 μg of selenium, and the mass spectrum was obtained with 0.4 μg of selenium (equivalent to about 0.12 mg of GSH peroxidase).

Denaturation with guanidine is essential for derivatization of the active site selenium of GSH peroxidase. In preliminary experiments where guanidine was not used, the borohydride-reduced enzyme was completely inhibited by FDNB within 30 min, but the yield of *Se*-DNP-selenocysteine after acid hydrolysis was only 10–14% of the enzyme selenium. In the presence of guanidine, close to 90% yield of *Se*-DNP-selenocysteine was obtained. Traces of other unidentified selenium compounds were observed during fractionation of the hydrolysate in addition to *Se*-DNP-selenocysteine. Some of these may arise by decomposition. Moreover, it is likely that preparations of enzyme that have become partially inactivated during isolation or storage will contain some portion of the selenium in a nonactive, degraded form such as elemental selenium. Such forms of selenium will either fail to be derivatized or, upon reduction, may be converted to other selenols such as hydrogen selenide that form DNP derivatives. Hydrogen selenide forms monoselenide and diselenide derivatives of DNP that are extractable into benzene and thus would be removed prior to chromatography.

The *Se*-DNP derivative of selenocysteine has the expected stability but with certain reservations. It is quite stable when stored under acidic conditions and if hydrolyzed under strictly anaerobic conditions. When hydrolysis was conducted on samples of *Se*-DNP-selenocysteine in an atmosphere of air, the recovery of Se was 64% in the aqueous phase and 34% in the benzene phase, whereas under nitrogen the comparable values were 95 and 5%. Under mildly basic conditions, decomposition by the intramolecular Se → N rearrangement of the DNP group takes place. This rearrangement process interfered with some initial attempts to apply mass spectroscopy to the *Se*-DNP-selenocysteine, either directly or after derivatization with various reagents to enhance the volatility. Invariably, selenium fragments were absent from the mass spectra, and selenium remained on the probe. After publication of the paper by Kondo et al. (1981) showing that *S*-DNP-cysteine underwent the S → N intramolecular shift (Smiles rearrangement), we obtained chromatographic evidence that a similar Se → N rearrangement occurred with *Se*-DNP-selenocysteine. Also, the presence of a *N*-DNP fragment (m/e = 209, CH_2–CH–NH–DNP) in the mass spectrum indicated that decomposition of the *Se*-DNP-selenocysteine on the probe occurred by this route, with loss of selenium from the selenol liberated during the rearrangement. We found the Smiles rearrangement could be used to advantage, if modified by the inclusion of methyl iodide to trap the selenol. The rearrangement of *Se*-DNP-selenocysteine in the presence of methyl iodide under anaerobic conditions formed the stable *Se*-methyl-*N*-DNP-selenocysteine in good yield:



The carboxylic acid was then esterified, and the ester was isolated by TLC and identified by mass spectroscopy. This rearrangement and esterification procedure was applied to the purified selenium isolated from the acid hydrolysate of FDNB-derivatized GSH peroxidase. The selenium from the enzyme had the same mass spectrum as the model compound prepared from either selenocystine or *Se*-methylselenocysteine.

The use of methyl iodide to trap the selenol intermediate produced during the Smiles rearrangement is a useful modification that stabilizes the rather labile–SeH group in the form of a selenoether. This derivative is not as susceptible to oxidation as the selenol and is less likely to undergo elimination compared to the DNP-Se derivative. Formation of the methylselenol also helps increase the volatility of the derivative for mass spectroscopy.

Although the present procedure using DNP derivatization was developed so that selenocysteine could be conclusively identified by mass spectroscopy, characterization based solely on cochromatography of the DNP derivative in high-resolution HPLC systems is even easier and could be adequate for some purposes, as in the case of other enzymes where FDNB has been used to derivatize amino acid residues at the active site (Swenson et al., 1982). However, the fact that N^{im}-DNP-histidine and DNP-arginine are eluted very similarly to *Se*-DNP-selenocysteine underlines the need for caution in using cocochromatography and the need for confirmation by mass spectroscopy. The procedure for derivatization for mass spectroscopy is quite simple. It is important, however, to have the *Se*-DNP-selenocysteine highly purified before performing the Smiles rearrangement. Contaminating substances can interfere with the intramolecular rearrangement through competing intermolecular reactions.

The identification of DNP-alanine in the benzene extract of the acid hydrolysate indicates that alanine is the N-terminal amino acid. DNP-alanine was the only acidic compound detected when the benzene extract was electrophoresed at pH 5.3, and it was conclusively identified by mass spectroscopy. The DNP derivatives of dicarboxylic amino acids (glutamic, aspartic) were not present in the benzene extract. The absence of di-DNP derivatives of lysine and histidine and *N*-DNP-arginine in the aqueous phase excludes the basic amino acids. Arginine was tentatively identified as the N-terminal amino acid in bovine erythrocyte GSH peroxidase on the basis of X-ray crystallography (Ladenstein et al., 1979). Glycine is believed to be the N-terminal amino acid of rat liver GSH peroxidase (Condell & Tappel, 1982).

Added in Proof

Recently, a refined structure for bovine erythrocyte GSH peroxidase at 0.2 nm has appeared (Epp et al., 1983). Alanine was reported to be the N-terminal amino acid for the bovine enzyme, in agreement with our results for the ovine enzyme.

Acknowledgments

The initial mass spectral analyses were done at the University of Wisconsin Food Research Institute by Dr. Walter Hargraves, and his cooperation is greatly appreciated. We thank Dr. Heinrich Schnoes and M. Micke of the Department of Biochemistry for obtaining the mass spectra shown in this paper. NMR spectra were obtained through the courtesy of Dr. H. Reich and C. A. Hoeger of the Department of Chemistry. We thank Dr. Vincent Massey of the University of Michigan for supplying a sample of N^{im}-DNP-histidine.

Registry No. Glutathione peroxidase, 9013-66-5; *Se*-DNP-selenocysteine, 87586-98-9; *Se*-methyl-*N*-DNP-selenocysteine, 87586-99-0; *S*-DNP-cysteine, 23815-63-6; N^2-acetyl-N^6-DNP-lysine,

22619-87-0; N^{ϵ}-DNP-lysine, 1094-76-4; selenocystine, 29621-88-3; cystine, 56-89-3; selenocysteine, 10236-58-5.

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Nucleotide Sequence of the *Dictyostelium discoideum* Small-Subunit Ribosomal Ribonucleic Acid Inferred from the Gene Sequence: Evolutionary Implications†

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ABSTRACT: We have determined the T_1 oligonucleotide catalog and the complete nucleotide sequence of the gene for the *Dictyostelium discoideum* small-subunit ribosomal RNA. The gene does not appear to contain an intron and has relatively low homology with other eukaryotic small-subunit rRNAs. The homology data indicate that *D. discoideum* diverged from the mainstream of eukaryotic descent at the earliest branch yet characterized by molecular phylogeny.

The deepest branchings within the eukaryotic line of descent are represented by members of the "kingdom" Protoctista.[1] It has not been possible to infer consistent phylogenies for these simple eukaryotes by using classical taxonomic approaches, i.e., comparative studies of phenotypes. This, in part, is a consequence of the enormous physiological, cytological, and biochemical diversity within the group.

Genealogical relationships between such diverse organisms can be inferred from comparisons of the amino acid or nucleotide sequences of functionally equivalent biopolymers (Zuckerkandl & Pauling, 1965). Analyses based upon amino acid sequence changes in cytochrome *c* (Fitch, 1976; McLaughlin & Dayhoff, 1973), ferredoxin (Ysunobu & Tanaka, 1973; Rao & Hall, 1977; Rao & Cammack, 1981), superoxide dismutase (Asada et al., 1980; Fridovich, 1976; Lumsden & Hall, 1975), etc. have proven extremely useful in confirming, refining, and extending classical eukaryotic phylogeny, but sometimes they produce unconvincing junctions at the deepest phylogenetic levels where their limits as molecular "chronometers" are approached. By comparison, the ribosomal RNAs seem to be better suited for revealing phylogenetic relationships. They are universally distributed and are functionally equivalent in all cells. In addition, the size and the conservative nature of large portions of the 16-18S and 23-28S rRNAs permit the measurement of the largest phylogenetic distances (Stackebrandt & Woese, 1981; Otsuka et al., 1983). Yet, other regions of the molecule change more rapidly, and this allows the measurement of close phylogenetic relationships. Finally, the sequence homology that exists between the rRNAs of the eukaryotes and both kingdoms of the prokaryotes permits the evaluation of relative rates of

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[1] Membership in this division is "defined by exclusion: its members are neither animals (which develop from blastula), plants (which develop from an embryo), fungi (which lack undulipodia and develop from spores), nor prokaryotes" (Margulis & Schwartz, 1982).

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