

BBA 91186

IN VITRO SYNTHESIS OF GLUTATHIONE PEROXIDASE FROM SELENITE

TRANSLATIONAL INCORPORATION OF SELENOCYSTEINE

WAYNE C. HAWKES and AL L. TAPPEL *

Department of Food Science and Technology, 1480 Chemistry Annex, University of California, Davis, CA 95616 (U.S.A.)

(Received August 19th, 1982)

(Revised manuscript received November 8th, 1982)

Key words: Glutathione peroxidase; tRNA; Selenocysteine, Selenite, Protein synthesis

The synthesis of glutathione peroxidase from [⁷⁵Se]selenite was studied in slices and cell-free extracts from rat liver. The incorporation of [⁷⁵Se]selenocysteine at the active site was detected by carboxymethylation and hydrolysis of partially purified glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) in the presence of [³H]selenocysteine and subsequent amino acid analysis. The synthesis of glutathione peroxidase in slices was inhibited by cycloheximide or puromycin and ⁷⁵Se was incorporated from [⁷⁵Se]selenite into free selenocysteine and selenocysteyl tRNA. Increasing concentrations of selenocysteine caused a progressive dilution of the ⁷⁵Se and a corresponding decrease in glutathione peroxidase labeling. In cell-free systems, [⁷⁵Se]selenocysteyl tRNA was the best substrate for glutathione peroxidase synthesis. These results indicate the existence in rat liver of the de novo synthesis of free selenocysteine and a translational pathway of selenocysteine incorporation into glutathione peroxidase

Introduction

Since 1959, when McConnell et al. [1] reported the occurrence of ⁷⁵Se-labeled mercapturic acids in dog urine after [⁷⁵Se]selenite injection, it has been speculated that mammals synthesize selenocysteine from inorganic selenium. Substantial weight was given to this idea by the work of Godwin and Fuss [2] and Olson and Palmer [3], who reported the presence of selenocysteine derivatives in amino acid analyzer chromatograms of pronase digests of ⁷⁵Se-labeled proteins from rabbit and rat tissues, respectively. Several selenium analogs of sulfur compounds are active substrates for sulfur-metabolizing enzymes [4–6], and Esaki and co-workers [7] have recently reported the in vitro

synthesis of selenocysteine from selenomethionine using purified enzymes from rat liver.

Those selenium-requiring proteins studied in sufficient detail, rat and bovine glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) contain the selenium as selenocysteine in an internal peptide linkage [8,9]. In fact, selenocysteine is the only form of selenium that has been reported in partially-characterized selenoproteins such as bacterial glycine reductase [10], bacterial formate dehydrogenase [11], a selenoprotein of ovine muscle [12], 'selenoflagellin' of rat sperm [13], and the numerous selenoproteins from rat tissues recently studied in this laboratory [14,15]. Although other forms of selenium have been reported in protein [16–18], those proteins which specifically require selenium have been found to contain selenocysteine.

Even though selenocysteine seems to be the

* To whom correspondence should be addressed.

most important form of selenium in protein, those organisms known to require selenium utilize inorganic selenium as selenite about as well as any other form. This indicates the presence of a metabolic pathway for the de novo synthesis of selenocysteine from selenite, but such a pathway has never been directly demonstrated in mammals.

Much of the recent work in selenium metabolism has centered on the mechanism by which selenocysteine is produced in a precise location in the polypeptide backbone of selenoenzymes such as glutathione peroxidase. Such mechanisms fall into two categories: post-translational modification of a pre-existing amino acid residue and incorporation of a selenoamino acid from tRNA during protein synthesis. Evidence has been sought for both types of pathway with conflicting results. Most researchers have concentrated on post-translational pathways. Isotope dilution studies in perfused rat liver by Sunde and Hoekstra [19] and in liver slices in this laboratory [20] indicated selenite is metabolically closer to glutathione peroxidase than is selenocystine, a finding that favors a post-translational pathway. However, Yoshida and co-workers [21] could find no evidence for an apoglutathione peroxidase using antibodies raised against the purified enzyme. Other work in this laboratory [22] has identified a selenium-specific, selenocysteyl-aminoacyl tRNA in rat liver slices incubated with [^{75}Se]selenocysteine. This work suggested that a translational pathway of glutathione peroxidase synthesis may exist in rat liver. The purpose of the present investigation was to find if such a translational pathway exists in rat liver and whether it is the normal pathway of glutathione peroxidase synthesis in rats.

Materials and Methods

Materials. [^{75}Se]Selenious acid (9–35 Ci/mmol) was purchased from New England Nuclear and L-[^{75}Se]selenocystine (150–300 mCi/mmol) from Amersham Radiochemical Center. D,L-[^3H]selenocystine (50 mCi/mmol) was prepared by Amersham via custom tritiation of unlabeled selenocystine and subsequent purification on an amino acid analyzer. Rats were male albinos of the Sprague-Dawley strain (Simonsen Laboratories) that weighed 60–150 g at death. DEAE-Sephacel

was purchased from Sigma and Sepharose 4B from Pharmacia Fine Chemicals.

Liver slice incubations. The rats were killed by decapitation and bled. Livers were rapidly excised and immersed in ice-cold Hank's balanced salt solution. Slices of 0.8 mm thickness were prepared on a McIllwain tissue chopper, washed in cold Hank's balanced salt solution, blotted and weighed in a tared container of cold Hank's balanced salt solution. The liver slices were placed in Erlenmeyer flasks with 4 or 5 ml of 1 μM [^{75}Se]selenite-Hank's balanced salt solution/g of slices. The flasks were aerated for 1 min with oxygen, sealed and placed in a shaker bath at 37°C.

Glutathione peroxidase purification. Washed liver slices were homogenized 1:4 (w/v) for 90 s in 10 mM Tris-HCl (pH 7.6)/0.1 mM EDTA/0.25 M sucrose in a glass and Teflon homogenizer. The homogenates were centrifuged at 100 000 $\times g$ for 60 min, and 5 μl mercaptoethanol/g of slices was added to the supernatants. The supernatants were heated to 50°C for 45 min and then cooled on ice before addition of 6 ml of cold acetone/g of slices. After allowing the mixtures to stand for at least 15 min at -15°C , they were centrifuged at 15 000 $\times g$ for 15 min. The pellets were resuspended in 2 ml 10 mM Tris-HCl (pH 7.6)/0.1 mM EDTA/5 mM mercaptoethanol (Buffer A)/g of slices and then centrifuged at 15 000 $\times g$ for 15 min. The supernatants were applied to miniature columns of DEAE-Sephacel (2 ml/g of slices) in Buffer A and eluted with 2 ml Buffer A/g of slices. Some of the eluates were concentrated in an Amicon Minicon-B ultrafiltration cell.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 7.5% SDS gels were prepared by the method of Wu and Breuning [23]. To each 45 μl of partially purified glutathione peroxidase was added 45 mg urea, 0.5 mg dithioerythritol, 23 μl mercaptoethanol, 7 μl 25% triethanolamine and 9 μl SDS (180 mg/ml). After heating the samples for 10 min in a boiling water bath, each was mixed with 2.7 μl 3 M HCl and 1.8 μl 1% Phenol red. After electrophoresis, the gels were sliced in 2 mm sections and counted for ^{75}Se . The total counts of ^{75}Se radioactivity at the position of glutathione peroxidase ($R_m = 0.88$) were divided by the total enzyme units of glutathione peroxidase loaded on the gel and then multiplied by the glutathione

peroxidase enzyme activity in the crude homogenate in order to calculate the total selenium incorporated into glutathione peroxidase per gram of slices per hour.

Selenocysteine trapping. Samples of freshly prepared crude homogenate were diluted 1:3 (v/v) with distilled water and mixed with 0.5 μ mol of carrier selenocystine. The samples were adjusted to 0.1 M Tris-HCl (pH 8.5), 20 mM sodium iodoacetate and 4 mg/ml sodium borohydride. A few drops of silicone antifoam emulsion were added and the samples were incubated with shaking for 20 min at 37°C. After cooling the samples on ice, 50% trichloroacetic acid was added to a concentration of 8%, and protein was precipitated for 20 min at 4°C. Solids were removed by filtration on Whatman No. 1 paper, and the filtrate was applied to a 5 ml column of AG-50 W-X8 (H^+ form in 0.1 M HCl). The column was washed with 10 ml of 0.1 M HCl and 20 ml of distilled water before the amino acids were eluted with 2 M HCl. The samples were concentrated by evaporation under vacuum, mixed with [3H]carboxymethylselenocysteine and loaded on a Beckman Model 120B amino acid analyzer. Amino acids were analyzed using a column of Durrum AA-20 resin and a five-buffer lithium citrate system programmed for physiological amino acids. 1 or 2 min fractions were collected and counted for radioactivity. When sufficient radioactivity was present, 3H and ^{75}Se were determined by dual-channel flow scintillation counting of the amino acid analyzer eluate.

Transfer RNA purification. Aminoacyl tRNA was purified by a slight modification of the method of Hawkes et al. [22] using multiple phenol extractions, 1 M NaCl extraction and chromatography on DEAE-Sephacel. The only change from the referenced procedure was the inclusion of 10 mM mercaptoethanol in all of the buffers. The crude aminoacyl tRNA was precipitated with ethanol, dissolved in 10 mM $MgCl_2$ /1 mM EDTA (pH 4.5) and stored in liquid nitrogen.

Cell-free protein synthesis. The livers from fasted, 80–100 g rats were homogenized in 2 vols. of freshly prepared 50 mM Tris-HCl (pH 7.8)/25 mM KCl/20 mM mercaptoethanol/10 mM $MgCl_2$ /4.3 mM phosphoenolpyruvate/0.5 mM ATP/0.12 mM GTP/0.375 mg/ml pyruvate

kinase. The homogenate was centrifuged for 10 min at 10 000 $\times g$, and the entire post-mitochondrial supernatant was decanted and saved. [^{75}Se]Selenocystine was reduced in 2 mg/ml KBH_4 (pH 8.5) for 5 min at 45°C. 80 μ l ^{75}Se -labeled tRNA or other labeled substrate was added to 1 ml of post-mitochondrial supernatant, and the samples were incubated for 60 min at 37°C. One-half of the incubation mixtures contained 1 mM cycloheximide to inhibit protein synthesis. At the end of the incubation period, 20 μ l 0.5 M sodium iodoacetate were added and the incubations were continued for 2 min at 37°C. The entire contents of each incubation mixture were treated with urea, SDS, mercaptoethanol, triethanolamine and dithioerythritol as described for SDS-gel samples. After boiling the mixtures for 10 min, Blue dextran and Cresol red dyes were added as markers, and the samples were chromatographed on a 1.5 \times 90 cm column of Sephacryl S300 in 0.2 M Tris-phosphate (pH 6.9)/7 M urea/5 mM SDS. The ^{75}Se at the elution position of a liver slice-labeled glutathione peroxidase standard (partition coefficient = 0.40) was summed and expressed as a percentage of the total ^{75}Se added to the incubation mixture.

Assays. Glutathione peroxidase was measured by the procedure of Stults et al. [24], which was modified by the addition of 10 mM NaCN to the preincubation mixture to inhibit the nonselenium-dependent glutathione peroxidase [25]. ^{75}Se was counted on a Packard Model 5210 Auto-Gamma NaI scintillation counter at 30% efficiency. 3H was counted on a Beckman LS-100 liquid scintillation counter at 21% efficiency. When both isotopes were present, ^{75}Se was first determined on the gamma counter, then $^3H + ^{75}Se$ was counted by liquid scintillation, and the counts contributed by ^{75}Se were subtracted from the total counts.

Results

Synthesis of glutathione peroxidase from selenite in liver slices

Glutathione peroxidase was partially purified from liver slices incubated for 60 min in 5 ml of [^{75}Se]selenite-Hank's balanced salt solution/g of slices as described in Materials and Methods. To show that the ^{75}Se from [^{75}Se]selenite was incorporated specifically into glutathione peroxidase, the

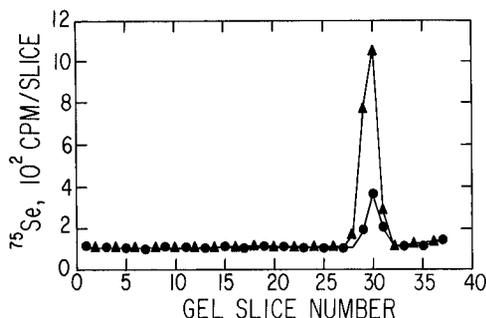


Fig. 1. SDS-gel electrophoresis of in vitro-labeled ^{75}Se -glutathione peroxidase (●) prepared as described in Materials and Methods and authentic ^{75}Se -glutathione peroxidase standard (▲) prepared by the method of Forstrom et al. [26]. Analysis was on 7.5% gels that contained 7 M urea.

electrophoretic mobility of in vitro-labeled glutathione peroxidase on SDS and discontinuous pH gels was compared to that of standard rat liver glutathione peroxidase prepared by the method of Forstrom et al. [26], and the two were indistinguishable (Figs. 1 and 2). The form of ^{75}Se in in vitro-labeled glutathione peroxidase was shown to be selenocysteine by hydrolyzing a carboxymethylated sample of the enzyme in the presence of [^3H]carboxymethylselenocysteine after SDS-gel electrophoresis. Amino acid analysis of the hydrolyzate showed that all of the ^{75}Se remaining in liver slice labeled glutathione peroxidase after SDS-gel electrophoresis was present as [^{75}Se]-carboxymethylselenocysteine.

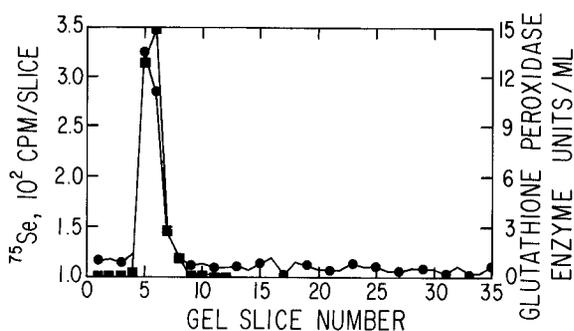


Fig. 2. Discontinuous-pH gel electrophoresis of in vitro-labeled ^{75}Se -glutathione peroxidase on 7.5% gels at pH 8.8 prepared by the methods of Ornstein [27] and Davis [28]. 2 mm slices were eluted overnight into 0.5 ml Buffer A, and the supernatant was assayed for ^{75}Se (●) and glutathione peroxidase activity (■).

The effect of protein synthesis inhibitors and unlabeled selenocysteine on glutathione peroxidase labeling is shown in Table I. The protein synthesis inhibitors puromycin and cycloheximide were both potent inhibitors of the incorporation of ^{75}Se from selenite into glutathione peroxidase, showing that the ^{75}Se was incorporated into newly synthesized protein. The increasing inhibition of glutathione peroxidase labeling with increasing concentrations of selenocysteine is consistent with dilution of ^{75}Se in an intracellular pool of a selenocysteine derivative. That this effect of selenocysteine was not due to a general inhibition of cellular metabolism was shown by measuring the evolution of $^{14}\text{CO}_2$ from [^{14}C]glucose in liver slices with or without 5 μM selenocysteine. In triplicate pairs of incubations no effect of selenocysteine was seen on $^{14}\text{CO}_2$ evolution (data not shown). That the effect of selenocysteine was not due to inhibition of protein synthesis was shown by measuring the incorporation of [^{35}S]methionine into protein in a cell-free system (as described in Materials and Methods) with and without 40 μM selenocysteine. Triplicate

TABLE I

INHIBITION OF GLUTATHIONE PEROXIDASE SYNTHESIS IN LIVER SLICES

1 g of liver slices was incubated for 60 min in 5 ml of [^{75}Se]selenite-Hank's balanced salt solution that contained the indicated additions as described in Materials and Methods. The data shown are the means of three samples \pm S.E. Since aliquots of slices from each of three livers were tested in all five conditions, the groups were compared to controls using a paired t test. All groups were significantly different from the controls ($P < 0.005$).

Addition	Concentration	^{75}Se -Glutathione peroxidase synthesis rate	
		(pmol/h per g)	(% of control)
Control	-	2.20	100
Puromycin	1 mM	0.40	18.2 \pm 0.6 ^a
Cycloheximide	1 mM	0.31	14.2 \pm 7.7
Selenocysteine	3 μM	1.10	49.3 \pm 16.3
			$P < 0.01$
Selenocysteine	5 μM	0.23	10.6 \pm 2.0

^a Mean of two determinations \pm one-half the range.

samples showed no effect of selenocystine on [^{35}S]methionine incorporation (data not shown). The effect of selenocystine on glutathione peroxidase synthesis from selenite is therefore a specific effect on selenium metabolism.

Synthesis of free selenocystine from selenite in liver slices

The acetone supernatant from a glutathione peroxidase purification was used initially as the source of ^{75}Se -labeled amino acids to search for the presence of free [^{75}Se]selenocystine. The supernatant was stored for 2 weeks at -15°C , concentrated by evaporation under vacuum at 37°C , then mixed with [^3H]selenocystine and an equal volume of 6% 5'-sulfosalicylic acid to remove protein. Amino acid analysis showed no co-elution of ^{75}Se and ^3H at the position of selenocystine or selenocystine. Next, the concentrated acetone supernatant was mixed with [^3H]selenocystine and treated with potassium borohydride before amino acid analysis. Under these conditions, the chromatogram again showed no coincidence of ^{75}Se and ^3H . The only successful demonstration of the presence of a form of free selenocystine was obtained when carboxymethylselenocystine was isolated from freshly incubated liver slices as described in Materials and Methods. In several experiments, from 2 to 6-times as much ^{75}Se was found in carboxymethylselenocystine as was found in glutathione peroxidase (Table II), which indicated that some form of free selenocystine

was synthesized from selenite in rat liver slices. Since the presence of [^{75}Se]selenocystine could only be detected using rapid isolation and reduction with borohydride, the native form of selenocystine must have been more oxidized than the selenol. The requirement to trap the selenocystine as carboxymethylselenocystine indicates the lability of the selenol form under these conditions.

Synthesis of selenocysteyl-aminoacyl tRNA from selenite in liver slices

Liver slices were incubated for 30 min in 4 ml of [^{75}Se]selenite-Hank's balanced salt solution/g of slices as described in Materials and Methods except 20 amino acids were added to the incubation buffer at $40\ \mu\text{M}$ each. Of the total ^{75}Se absorbed by the slices (Table II), 0.39% was recovered in the purified tRNA fraction, which corresponded to a charging level of approx. 0.03%. Glutathione peroxidase purified from an aliquot of ^{75}Se -labeled slices from the same incubation contained an amount of ^{75}Se equivalent to 0.09% of the total absorbed ^{75}Se . All of the ^{75}Se -labeled tRNA eluted as a single peak on Sepharose 4B at an ammonium sulfate concentration of 0.96 M, the same elution position previously observed for selenocysteyl-aminoacyl tRNA made from selenocystine in liver slices [22]. 93% of the ^{75}Se in tRNA was removed by incubation for 20 min at 37°C in 0.3 M Tris-HCl (pH 8.5). Of the ^{75}Se released from tRNA by mild alkaline hydrolysis, 16.9% was recovered as selenocystine after amino acid analysis (Fig. 3A). The deacylated ^{75}Se was also treated with potassium borohydride and iodoacetate or iodoacetamide, in the presence of [^3H]selenocystine. Treatment of the deacylated ^{75}Se with iodoacetate (Fig. 3B) resulted in a chromatogram showing coincidence of ^{75}Se and ^3H in three compounds; selenocystine, selenocystine and carboxymethylselenocystine. The chromatogram of the iodoacetamide-treated deacylate (Fig. 3C) showed coincidence of ^{75}Se and ^3H in two other compounds, carboxamidomethylselenocystine and an unidentified derivative. When the ^{75}Se in these ^3H -containing peaks was corrected for the partial ^3H recoveries, from 9.3–14.0% of the ^{75}Se on tRNA was shown to have been in a form of selenocystine. Since these values were lower than the recovery of selenocystine from the untreated

TABLE II
DISTRIBUTION OF ^{75}Se IN SELENIUM COMPOUNDS AFTER LIVER SLICE INCUBATION WITH [^{75}Se]SELENITE

The values shown are typical results taken from several experiments. 2 g of liver slices were incubated for 30 min in 10 ml of $1\ \mu\text{M}$ [^{75}Se]selenite-Hank's balanced salt solution as described in Materials and Methods.

Fraction	Total Se (pmol)	Percentage of absorbed Se
Total incubation	10 000	—
Homogenate	2 500	100
Carboxymethylselenocystine	4.5	0.18
Selenocysteyl tRNA	9.8	0.39
Glutathione peroxidase	2.1	0.09

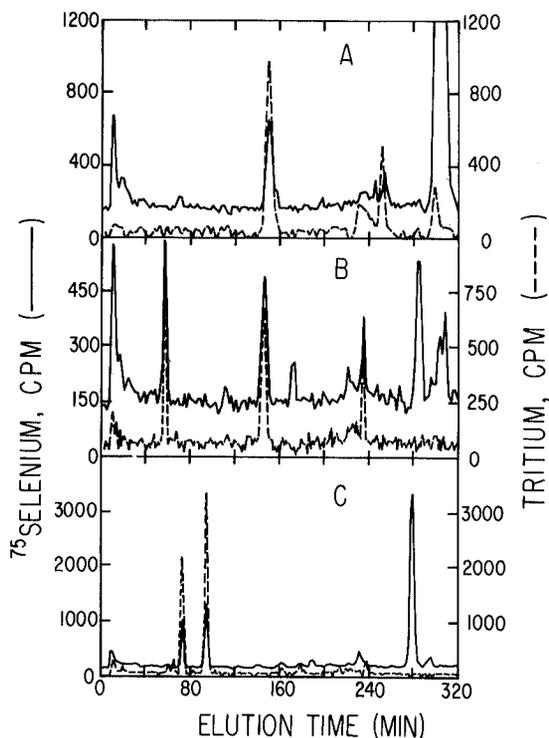


Fig. 3. Amino acid analyzer chromatograms of deacylated ^{75}Se -labeled compounds from [^{75}Se]selenite-labeled tRNA. The tRNA was mixed with [^3H]selenocystine in 2 mg potassium borohydride/ml, then deacylated. Nucleic acid was precipitated with 3% 5'-sulfosalicylic acid. (A) Untreated deacylated ^{75}Se -labeled compounds, (B) deacylated ^{75}Se -labeled compounds treated with iodoacetate and (C) deacylated ^{75}Se -labeled compounds treated with iodoacetamide.

TABLE III

CELL-FREE SYNTHESIS OF GLUTATHIONE PEROXIDASE

The indicated ^{75}Se -labeled compounds were incubated for 60 min in 1 ml of a supplemented, post-mitochondrial rat liver supernatant as described in Materials and Methods. The data shown are the means \pm S.E. The number of determinations is shown in parentheses. n.s., not significant. SDS-S300, SDS chromatography on Sephacryl S-300.

Radioactive source	Total ^{75}Se (cpm)	Incorporation into $K = 0.40$ peak on SDS-S300		
		Without cycloheximide (%)	With cycloheximide (%)	Cycloheximide sensitive (%)
[^{75}Se]Selenocysteyl tRNA (4)	40000	4.96 ± 0.40	1.25 ± 0.08	3.70 ± 0.41 $P < 0.0001$
Selenocysteine (4)	80000	1.55 ± 0.42	0.80 ± 0.05	0.75 ± 0.42 $P < 0.1$
Deacylated [^{75}Se]selenocysteyl tRNA (3)	80000	1.82 ± 0.51	1.56 ± 0.07	0.26 ± 0.52 (n.s.)
Selenite (4)	400000	0.89 ± 0.12	0.77 ± 0.10	0.11 ± 0.16 (n.s.)

samples, all of the selenocysteine on tRNA must have already been in the selenol form.

Cell-free synthesis of glutathione peroxidase from selenocysteyl tRNA

The incorporation of ^{75}Se from [^{75}Se]selenocysteyl tRNA into glutathione peroxidase in post-mitochondrial supernatants was compared to that from [^{75}Se]selenocysteine, [^{75}Se]selenite and [^{75}Se]selenocysteyl tRNA that had been preincubated for 20 min at 37°C in 0.1 M Tris-HCl (pH 8.5) (Table III). Identical incubations were conducted with and without cycloheximide, and the net rates of cycloheximide-sensitive incorporation into glutathione peroxidase, expressed as a percentage of the total ^{75}Se , are given in the last column of Table III. These data show clearly that selenocysteyl tRNA is the best substrate for the synthesis of glutathione peroxidase and that selenocysteine is transferred from tRNA to glutathione peroxidase during protein synthesis. The low cycloheximide-sensitive activity of selenite is difficult to evaluate because its reactivity with thiols results in a variety of products [29]. It would have been preferable to compare these substrates in the absence of thiol, however, there are no thiol-independent mammalian protein synthesis systems known. Furthermore, selenite incorporation into glutathione peroxidase proceeds well in liver slices and perfused liver where the intracellu-

lar glutathione concentrations are 5–12 mM [30]. In order to ensure that the relatively high concentration of selenocysteine (1 μ M vs. 0.12 μ M [75 Se]selenite) was not inhibiting 75 Se incorporation into glutathione peroxidase, cell-free protein synthesis incubations were carried out with [75 Se]selenocysteyl tRNA in the presence of 1 μ M unlabeled selenocysteine (data not shown). The level of glutathione peroxidase labeling was the same in the presence of selenocysteine as it was in the controls.

If selenium is incorporated into glutathione peroxidase via a translational pathway *in vivo*, then it would be expected that selenocysteyl tRNA would be the best substrate for the cycloheximide-sensitive synthesis of glutathione peroxidase in a cell-free system. That this high level of incorporation was due to the activated aminoacyl linkage was shown by the drastically reduced incorporation level ($P < 0.005$) and the loss of cycloheximide sensitivity when the selenocysteyl tRNA was preincubated at pH 8.5. The fact that selenocysteine was a better substrate for cycloheximide-sensitive glutathione peroxidase synthesis than selenite ($P < 0.01$) suggests that selenocysteine is metabolically closer to glutathione peroxidase. This result does not agree with isotope dilution experiments in perfused liver by Sunde and Hoekstra [19] and with liver slices in this laboratory [20]. In these systems, [75 Se]selenite was the best substrate for glutathione peroxidase synthesis. However, in neither study was an allowance made for the effect of total selenium concentration or the effect of transport of the 75 Se-labeled compound and the unlabeled diluents into the liver. Because of these uncontrolled features of isotope dilution experiments in slices and whole organs, the results obtained by direct incorporation of selenium into glutathione peroxidase in a cell-free system are of much greater significance. To a first approximation, the order of the 75 Se-labeled compounds with respect to their cycloheximide-sensitive incorporation rates should reflect their relative position in the pathway of selenium incorporation.

That the cell-free incorporation of 75 Se from tRNA into the glutathione peroxidase subunit ($K = 0.40$) was due to specific incorporation of selenium was shown by comparing the SDS-S300 chromatograms of proteins labeled by seleno-

cysteyl tRNA to those of proteins labeled with [35 S]cysteine in the cell-free system described in Materials and Methods (Fig. 4). Although both chromatograms contained similar peaks of radioactivity at the void and at $K = 0.29$, there was no peak of 35 S at $K = 0.40$, showing that the glutathione peroxidase incorporation was not due to random incorporation of selenocysteine in place of cysteine.

The 75 Se peaks at $K = 0.40$ from [75 Se]selenite-labeled and [75 Se]selenocysteyl tRNA-labeled proteins were pooled separately, dialyzed exhaustively against distilled water and hydrolyzed in the presence of [3 H]carboxymethylselenocysteine. The content of [75 Se]selenocysteine was measured by amino acid analysis. Table IV summarizes this information for the various *in vitro* glutathione peroxidase synthesis systems studied in this investigation with some *in vivo* results from other workers. It seems clear from previous work that the *in vivo* pathway of glutathione peroxidase synthesis from selenite is characterized by complete inhibition by cycloheximide [32] and complete recovery of 75 Se as carboxymethylselenocysteine [26]. By these criteria, it is apparent that in liver slices selenite follows the same pathway as *in vivo*, albeit at a reduced rate. In contrast, the labeling of glutathione peroxidase from selenite in

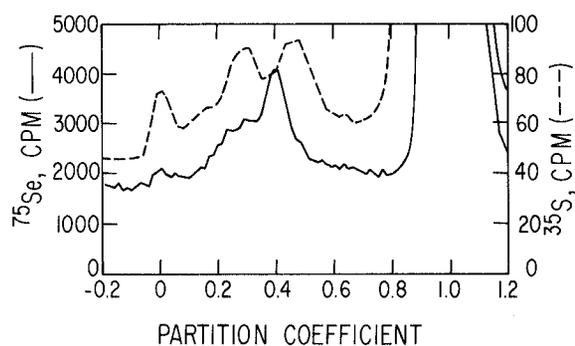


Fig. 4. Sephacryl S-300 chromatography of SDS subunits from cell-free protein synthesis with [75 Se]selenocysteyl tRNA or [35 S]cysteine. The proteins were labeled in the post-mitochondrial supernatant system described in Materials and Methods. The eluting buffer was 0.2 M Tris-phosphate (pH 6.9)/7 M urea/5 mM SDS. Blue dextran and Cresol red dyes were added to each sample as markers for the column void and total volumes, respectively. The partition coefficient was defined as $K = (V_c - V_0)/(V_t - V_0)$.

in the liver slices, which resulted in a uselessly low specific radioactivity. Since free selenocysteine occurs only in an oxidized form, an intriguing possibility is that the synthesis of free 'oxidized selenocysteine' might involve substitution by selenite for an acetyl or a phosphate group on serine. Further work is needed to demonstrate the feasibility of such a reaction. A recent report [34] concludes that rat liver selenocysteine is produced by reversal of the selenocysteine-lyase reaction: selenocysteine \rightarrow H₂Se + alanine.

Some previous work in this area raised questions about selenocysteine being an intermediate in glutathione peroxidase synthesis from selenite: isotope dilution with selenocysteine (diselenide) in liver slices [20] and perfused liver [19] suggested that selenite was metabolically closer to glutathione peroxidase than was selenocystine, and relative incorporation rates into glutathione peroxidase in liver slices showed that selenite was a better substrate than selenocystine [20]. An explanation for these apparently contradictory results can be found by noting that the selenium compound used in both studies was the diselenide, selenocystine, while the present results show that the actual form of selenocysteine in liver slices is some other oxidized derivative. Therefore, it is reasonable to find that selenocystine is neither an intermediate in, nor a good substrate for, the synthesis of glutathione peroxidase. Less obvious is why an oxidized form of selenocysteine is made when the products (tRNA and glutathione peroxidase) contain the selenocysteine as the selenol. One possibility is that the appreciable activity of selenocysteine lyase in rat liver [34] could be the reason that free selenocysteine occurs mostly in an oxidized form, thus preventing its degradation to hydrogen selenide by the lyase. The identity of the nonselenocysteyl ⁷⁵Se on tRNA as a degradation product of selenocysteine is suggested by the fact that freshly prepared [⁷⁵Se]selenocysteine had a measurable cycloheximide-sensitive incorporation rate in the cell-free protein synthesis system, while the ⁷⁵Se from deacylated tRNA did not (Table III). The greater activity of the selenocysteine implies that the deacylated ⁷⁵Se had been at least partially degraded. This interpretation is further supported by the fact that not all of the ⁷⁵Se incorporated into glutathione peroxidase from

tRNA could be recovered as carboxymethylselenocysteine (Table IV). The fact that the recovery of selenocysteine from the tRNA-labeled glutathione peroxidase was greater than its abundance on tRNA (40 vs. 16.9%) shows that the tRNA charged with selenocysteine is used for protein synthesis preferentially over the same tRNA charged with other forms of ⁷⁵Se. If the cycloheximide-sensitive [⁷⁵Se]selenocysteine incorporation into all molecular weights of protein is considered, it can be calculated that about one-half of the [⁷⁵Se]selenocysteine on tRNA was transferred into protein. Such efficient incorporation is only observed when aminoacyl tRNAs are the substrates. The substrate specificity and molecular properties of the aminoacyl tRNA synthetase that catalyzes the formation of selenocysteyl tRNA should be of great value in furthering the understanding of this aspect of the pathway.

The incorporation of selenocysteine from tRNA at the active site of glutathione peroxidase implies that there is a codon for selenocysteine. Although all 64 triplet codons have been shown to have some type of biological activity, the assays used were never tested with selenocysteine. For example, any codon in a protein synthesis assay would be interpreted as a termination codon in the absence of its specific amino acid. Furthermore, it has been reported that the termination codon U-G-A is specific for cysteine in porcine liver and *Xenopus* [35]. In addition, a phosphoseryl tRNA from bovine liver has recently been shown to respond to U-G-A in ribosomal binding assays and to repress U-G-A termination in in vitro assays [36]. It seems possible, therefore, that selenocysteyl tRNA could be coded for by a heretofore unrecognized codon. The fact that A-U-G can code for two different tRNAs, depending on its position in the message, seems to allow that U-G-A or another codon could have such a dual role with selenocysteine. We are presently searching for the putative selenocysteine codon by following the incorporation of [⁷⁵Se]selenocysteine into protein in response to synthetic mRNAs. Eventually, ribosomal binding of the labeled tRNA and sequencing of the anticodon could be used to make a final codon assignment.

In conclusion, these results show that rat liver can accomplish the de novo synthesis of selenocys-

steine from selenite and that selenocysteine can be incorporated into glutathione peroxidase via an aminoacyl tRNA. This demonstration of a translational pathway of glutathione peroxidase synthesis in rat liver would seem to rule out any significant role of post-translational mechanisms in the incorporation of selenium into glutathione peroxidase. Any future claim of the involvement of such a mechanism would have to be based on a demonstration of the existence of an apoenzyme of glutathione peroxidase. Lacking any such evidence, we are forced to conclude that the physiologically significant pathway of selenium incorporation into glutathione peroxidase is via selenocysteyl tRNA and protein synthesis.

Acknowledgement

This research was supported by research grant AM-06424 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

References

- 1 McConnell, K.P., Kreamer, A.E. and Roth, D.M. (1959) *J. Biol. Chem.* 234, 2932-2934
- 2 Godwin, K.O. and Fuss, C.N. (1972) *Aust. J. Biol. Sci.* 25, 865-871
- 3 Olson, O.E. and Palmer, I.S. (1976) *Metabolism* 25, 299-306
- 4 Wilson, L.G. and Bandurski, R.S. (1958) *J. Biol. Chem.* 233, 975-981
- 5 Hoffman, J.L., McConnell, K.P. and Carpenter, D.R. (1970) *Biochim. Biophys. Acta* 199, 531-534
- 6 Pan, F. and Tarver, H. (1967) *Arch. Biochem. Biophys.* 119, 429-434
- 7 Esaki, N., Nakamura, T., Tanaka, H., Susuki, T., Morino, Y. and Soda, K. (1981) *Biochemistry* 20, 4492-4496
- 8 Zakowski, J.J., Forstrom, J.W., Condell, R.A. and Tappel, A.L. (1978) *Biochem. Biophys. Res. Commun.* 84, 248-253
- 9 Ladenstein, R., Epp, O., Bartels, K., Jones, A., Huber, R. and Wendel, A. (1979) *J. Mol. Biol.* 134, 199-218
- 10 Cone, J.E., Martin del Rio, R., Davis, J.N. and Stadtman, T.C. (1976) *Biochemistry* 73, 2659-2663
- 11 Jones, J.B., Dilworth, G.L. and Stadtman, T.C. (1979) *Arch. Biochem. Biophys.* 195, 255-260
- 12 Beilstein, M.A., Tripp, M.J. and Whanger, P.D. (1980) *Fed. Proc.* 39, 338
- 13 Calvin, H.I., Wallace, E. and Cooper, G.W. (1981) in *Selenium in Biology and Medicine* (Spallholz, J.E., Martin, J.L. and Ganther, H.E., eds.), pp. 319-324, Avi Publishing Co., Westport, CT
- 14 Wilhelmsen, E.C., Hawkes, W.C., Motsenbocker, M.A. and Tappel, A.L. (1981) in *Selenium in Biology and Medicine* (Spallholz, J.E., Martin, J.L. and Ganther, H.E., eds.), pp. 535-539, Avi Publishing Co., Westport, CT
- 15 Motsenbocker, M.A. and Tappel, A.L. (1982) *Biochim. Biophys. Acta* 704, 253-260
- 16 Jenkins, K.J. (1968) *Can. J. Biochem.* 46, 1417-1425
- 17 Huber, R.E. and Criddle, R.S. (1967) *Biochim. Biophys. Acta* 141, 587-599
- 18 Butler, G.W. and Peterson, P.J. (1967) *Aust. J. Biol. Sci.* 20, 77-86
- 19 Sunde, R.A. and Hoekstra, W.G. (1981) in *Selenium in Biology and Medicine* (Spallholz, J.E., Martin, J.L. and Ganther, H.E., eds.), pp. 454-459, Avi Publishing Co., Westport, CT
- 20 Hawkes, W.C. (1980) Ph. D. thesis, University of California, Davis, CA
- 21 Yoshida, M., Iwami, K. and Yasumoto, K. (1982) *Agric. Biol. Chem.* 46, 41-46
- 22 Hawkes, W., Lyons, D. and Tappel, A. (1982) *Biochim. Biophys. Acta* 699, 183-191
- 23 Wu, G.J. and Breuning, G. (1971) *Virology* 46, 596-612
- 24 Stults, F.H., Forstrom, J.W., Chiu, D.T.Y. and Tappel, A.L. (1977) *Arch. Biochem. Biophys.* 183, 490-497
- 25 Pierce, S. and Tappel, A.L. (1978) *Biochim. Biophys. Acta* 523, 27-36
- 26 Forstrom, J.W., Zakowski, J.J. and Tappel, A.L. (1978) *Biochemistry* 17, 2639-2644
- 27 Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* 121, 321-349
- 28 Davis, B.J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404-427
- 29 Ganther, H.E. (1968) *Biochemistry* 7, 2898-2905
- 30 Tateishi, N. and Higashi, T. (1978) in *Functions of Glutathione in Liver and Kidney* (Sies, H. and Wendel, A., eds.), pp. 3-7, Springer-Verlag, Berlin, Heidelberg, Germany
- 31 Lyons, D.E. (1981) Ph. D. thesis, University of California, Davis, CA
- 32 Sunde, R.A. and Hoekstra, W.G. (1980) *Fed. Proc.* 165, 291-297
- 33 Condell, R.A. and Tappel, A.L. (1982) *Biochim. Biophys. Acta* 709, 304-309
- 34 Esaki, N., Nakamura, T., Tanaka, H. and Soda, K. (1982) *J. Biol. Chem.* 257, 4386-4391
- 35 Nirenberg, M., Caskey, T., Marshall, R., Brimacombe, R., Kellogg, D., Doctor, B., Hatfield, D., Levin, J., Rottman, F., Pestka, S., Wilcox, M. and Anderson, F. (1966) *Cold Spring Harbor Symposia on Quantitative Biology* 31, 11-24
- 36 Diamond, A. and Dudock, B. (1982) *Fed. Proc.* 41, 1031