Volume 232, number 1, 243–248 FEB 05869 May 1988

The conversion of phosphoserine residues to selenocysteine residues on an opal suppressor tRNA and casein

Takaharu Mizutani and Teruaki Hitaka

Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan

Received 13 February 1988; revised version received 24 March 1988

This study has been undertaken in order to elucidate the mechanisms of incorporation of Se into glutathione peroxidase (GSHPx), in which selenocysteine corresponds to the opal termination codon UGA on the mRNA. We studied the above mechanisms using an opal suppressor tRNA, prepared from bovine liver, and casein as a model protein for the GSHPx apo-enzyme which might contain phosphoserine. The results showed that opal suppressor tRNA did not accept selenocysteine (lower than 0.1 mmol/mol) under the standard conditions. A trace amount of phosphoseryl-tRNA was converted to selenocysteyl-tRNA by incubation with H_2Se and some enzymes. Meanwhile, a number of phosphoserine residues in casein were converted to selenocysteine residues by incubation with H_2Se and enzymes. These results suggest that opal suppressor tRNA plays a role in synthesizing GSHPx via co- and/or post-translational mechanisms.

Suppressor tRNA; Glutathione peroxidase; Selenocysteine; Opal termination codon; Phosphoseryl-tRNA

1. INTRODUCTION

Glutathione peroxidase (GSHPx) plays an important role in the detoxification of hydrogen peroxide, organic hydroperoxides and lipid peroxides. Selenium is present in the form of selenocysteine (SeCys) at the active site of this enzyme [1,2]. There are two possible mechanisms for incorporation of SeCys at a precise location in the polypeptide backbone of GSHPx. One is a co-translational pathway, in which a tRNA accepts SeCys by the specific SeCys-tRNA synthetase [3] and SeCys on the tRNA is incorporated into GSHPx by the normal protein synthesis mechanism on ribosomes [4]. SeCys in formate dehydrogenase from *Escherichia coli* is inserted via a co-translational mechanism [5]. Another possibility is a post-translational mechanism, having been elucidated from results using perfused rat liver [6,7]. The results showed that the carbon source for the SeCys skeleton was provided by serine. The specific labeling of SeCys with ^{14}C from serine indicated that serine was the carbon source for SeCys. The specific activity is formed after incorporation of serine into GSHPx. Meanwhile, it is known that approx. 30% of the total bound Se in the body is found in GSHPx and that selenite is better than selenomethionine for incorporation of Se into GSHPx. The in vivo synthesis of SeCys is not clear in mammals.

The GSHPx gene has been sequenced and the codon for SeCys of the active site is UGA, the opal termination codon [8]. Meanwhile, there is only one tRNA species corresponding to UGA in mammals [9] and this tRNA accepts serine. This Ser-tRNA is phosphorylated by tRNA kinase [10], the phosphoserine (Ps) on Ps-tRNA being incorporated into phosphoprotein [11]. In a previous report, we showed that suppressor tRNA has a 150-fold weaker affinity for UGA than release factor [12]. Therefore, this suppressor tRNA does not play a suppressor role under normal conditions and the termination codon UGA is terminated by the release factor, except for some mRNAs which have a context structure near the UGA codon such as GSHPx mRNA. It has been discussed that this context effect arises from base pairing between a

Correspondence address: T. Mizutani, Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

5′-flanking and a 3′-flanking region near the codon UGA [7,13,14]. However, the general rule concerning this structure and the context effect has not been ascertained.

In order to understand the role of mammalian opal suppressor tRNA which may be related to the incorporation of SeCys into GSHPx, we studied the three steps of protein synthesis relating to suppressor tRNA; the first is a search for tRNA which can accept SeCys, followed by conversion of Ps-tRNA to SeCys-tRNA, and finally conversion of Ps-protein to SeCys-protein. Here, H_2Se, produced from selenite and glutathione reductase, was used as the source of Se for incorporation into tRNA or proteins.

2. EXPERIMENTAL

Suppressor tRNA, SerRS and tRNA kinase were prepared from bovine liver according to [10,15]. Crude enzyme preparations for incorporation of Se were the supernatant (enzyme A) at 8000 × *g* of mouse liver homogenates, the supernatant at 105000 × *g* (enzyme B), and the eluate at 0.3 M KCl from DEAE-cellulose chromatography of the supernatant at 105000 × *g* (cnzyme C). H_2Se, as Se source, was produced by a mixture of 10 μM selenite, 0.2 mM NADPH, 20 mM mercaptoethanol, 8 mM glutathione and 10 mM Hepes at pH 7.5 and 5 μg/ml glutathione reductase from yeast (Boehringer) [16]. The phosphoprotein, used as a standard protein containing Ps, was milk casein. Alkaline phosphatase from calf intestine was a product of Boehringer. L-Selenocystine was a generous gift from Professor Kenji Soda of Kyoto University. Selenocysteine was produced from selenocystine by reduction in a mercaptoethanol solution. Carboxymethylation of proteins was performed with iodoacetic acid at neutral pH. Analysis of amino acids was performed through thin-layer chromatography on silica gel G with phenol/water (3:1) and *n*-butanol/acetic acid/water (4:1:1). The amount of Se was determined by a fluorometric method using 2,3-diaminonaphthalene [17].

2.1. *Pathway 1 (co-translational); $tRNA^{su}$ + SeCys ⟶ SeCys-tRNA ⟶ GSHPx*

Suppressor tRNA (0.5 nmol) or crude bovine tRNA (10 mg) was incubated at 30°C for 30 min in a buffer (0.5 ml) comprising 0.2 M Hepes at pH 7.5, 20 mM $MgCl_2$, 20 mM KCl, 50 μM SeCys, 5 mM ATP, 10 mM mercaptoethanol and enzyme C (1.5 mg protein), in which SerRS and MetRS were active. The mixture was then chromatographed on Sephacryl S-200 (40 × 1.3 cm) in 10 mM acetate buffer (pH 4.6) and 10 mM mercaptoethanol to separate Se-tRNA from free Se compounds. The amount of Se in the eluates from the column was assayed.

2.2. *Pathway 2 (co-translational); $Ser\text{-}tRNA^{su}$ ⟶ Ps-tRNA ⟶ SeCys-tRNA ⟶ GSHPx*

The H_2Se solution (0.1 ml) previously described was mixed with 0.1 ml Ps-tRNA solution, prepared by incubation for 30 min at 30°C in a mixture containing suppressor tRNA (0.5 nmol), SerRS, tRNA kinase, [^{14}C]Ser (170 Ci/mol) and ATP in buffer [10]. Thereafter, 0.1 ml of 0.1 M Hepes (pH 6.4) was added to the H_2Se-tRNA mixture in order to stabilize Ps-tRNA, and 30 μl enzyme B or C was added to the mixture. The solution was incubated for 30 min at 30°C. Subsequently, the tRNA solution was acidified by addition of 0.2 ml of 1 M acetate buffer (pH 4.6) and chromatographed on Sephacryl S-200 in 10 mM acetate (pH 4.6) and 10 mM mercaptoethanol. The peak of amino[^{14}C]acyl-tRNA was collected by ethanol precipitation. The precipitate was washed with ethanol and dried. The tRNA was dissolved in 50 μl of 40 mM Tris base and 10 mM mercaptoethanol, followed by incubation for 30 min at 25°C to hydrolyze aminoacyl-tRNA to obtain free amino acids. ^{14}C-labeled amino acids, liberated from amino[^{14}C]acyl-tRNA, were recovered in the supernatant by ethanol precipitation of tRNA and analyzed by TLC. Authentic unlabeled SeCys was developed on the same plate. Meanwhile, in order to determine the amount of Se on aminoacyl-$tRNA^{su}$, unlabeled serine was used instead of [^{14}C]serine under the same experimental conditions.

2.3. *Pathway 3 (post-translational); $Ps\text{-}tRNA^{su}$ ⟶ Ps-GSHPx (apo-enzyme) ⟶ SeCys-GSHPx (mature)*

Incorporation of Ps via Ps-tRNA into phosphoprotein was observed, however the yield of phosphoprotein was low as described in our previous report [11]. Therefore, it is difficult to obtain sufficient amounts of Ps-GSHPx, in the above pathway, for analysis of the incorporation of Se into the apo-enzyme of GSHPx. We also do not have GSHPx mRNA at this stage. Thus, we continued the investigation by using milk casein as a model protein for Ps-GSHPx and studied the incorporation of Se into casein.

Casein (20 mg) was dissolved in 0.5 ml H_2Se solution. The reaction was initiated by the addition of 30 μl enzyme A or C, and then allowed to proceed for 2 or 24 h at 30°C. The mixture was chromatographed to remove free H_2Se on a Sephacryl S-200 column in a solution composed of 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5) and 10 mM mercaptoethanol. The amount of Se in the eluate from the column was determined. The casein fraction containing Se was dialyzed and lyophilized. Casein was dissolved in 5 mM mercaptoethanol and then reacted with 30 mM iodoacetic acid. Subsequently, carboxymethyl(CM)-casein was hydrolyzed in 6 N HCl at 100°C for 2 h. This hydrolyzate was analyzed by TLC. Silica on the plate was scraped off and the amount of Se in the scrapings was analyzed.

3. RESULTS

The results concerning pathways 1–3 are described sequentially. Fig.1A and B shows the chromatographic patterns on Sephacryl S-200 of crude and suppressor tRNAs incubated with enzyme C in the presence of SeCys, respectively. The amount of SeCys binding to crude tRNA (fig.1A) and $tRNA^{su}$ (fig.1B) was less than 0.1 mmol/mol tRNA, after subtraction of the basic amount of Se (1 ng/mg tRNA) in tRNA. Therefore, in pathway 1, the results show that $tRNA^{su}$ and crude tRNA

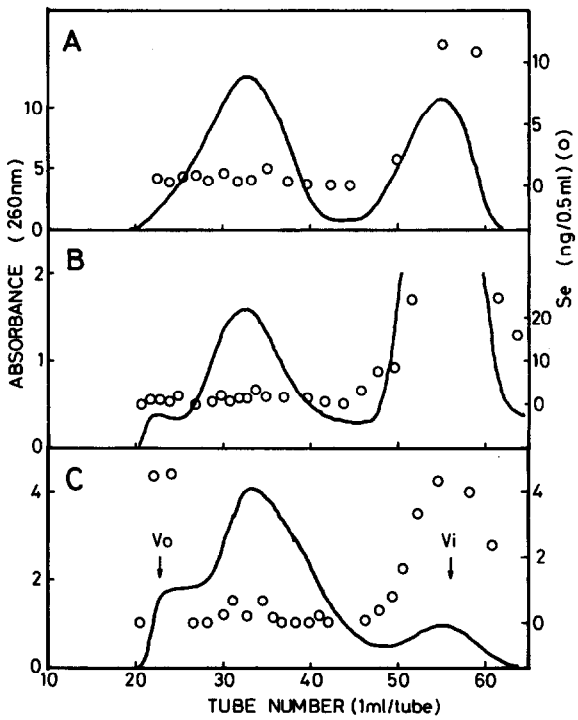


Fig.1. The chromatographic patterns of SeCys-tRNA on Sephacryl S-200 in 10 mM acetate buffer (pH 4.6) and 10 mM mercaptoethanol. (——) Absorbance at 260 nm; (○) amount of Se. (A) Crude tRNA; (B,C) suppressor tRNA; (B) tRNA incubated in the presence of SeCys, ATP and enzyme C; (C) Ps-tRNA incubated in the presence of H_2Se and enzyme C.

did not accept SeCys under the standard conditions. There remain two possible explanations for this lack of acceptance, namely that SeCys-tRNA synthetase is very labile, the other being that the amount of tRNA-specific SeCys present is very low in crude tRNA. Except for these two rare possibilities, the tRNA specific to SeCys is absent in mammals and suppressor tRNA does not accept SeCys.

We show next the results on pathway 2 (Ps-tRNA ⟶ SeCys-tRNA). Fig.1C depicts Sephacryl S-200 chromatography performed using the alcohol precipitate of the mixture of Ps-tRNA, H_2Se solution and enzyme C. The Se peak did not overlap with that of tRNA but was found at the shoulder (tubes 31–33) of the peak. The amount of Se bound on tRNA was 2 mmol/mol tRNA. The suppressor tRNA bound a small amount of Se. In order to confirm the presence of SeCys on aminoacyl-tRNA^{su}, [^{14}C]Ps-tRNA was incubated in H_2Se solution and isolated by chromatography on a Sephacryl S-200 column. The results of the analysis of ^{14}C-labeled amino acids liberated from the amino[^{14}C]acyl-tRNA are shown in fig.2. The radioactivity at the origin resulted from Ps. The major peak of R_f 0.20 was serine and the ^{14}C-labeled amino acid of R_f 0.32 should be alanine. One minor peak of ^{14}C was found at R_f 0.56. The percentage ratios of these amino acids were 8, 49, 14 and 17%, respectively. The amino acid at R_f 0.56 was not clear, however this position corresponds to aliphatic amino acids in the solvent of phenol/water (3:1). Authentic SeCys migrated to R_f 0.50 but we could not observe any radioactivity at the position of SeCys in fig.2. However, when a greater amount of [^{3}H]Ps-tRNA (some tens of thousands of cpm) was analyzed, a trace amount (0.1%) of total ^{3}H was clearly found at the position of authentic SeCys. Thus, we could not exclude the possibility of conversion of Ps-tRNA to SeCys-tRNA.

The results with respect to pathway 3 (phosphoprotein ⟶ SeCys-protein) were as follows. The chromatographic pattern for casein,

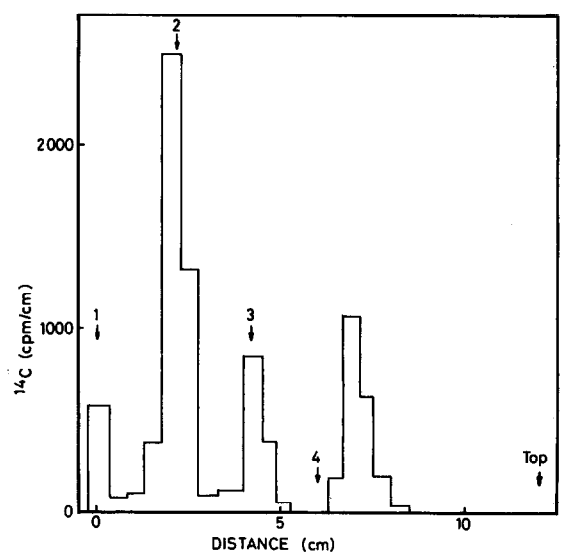
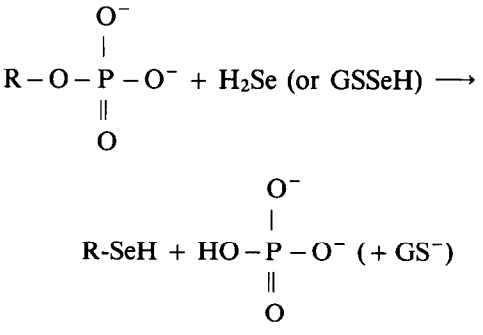


Fig.2. Analysis of ^{14}C-labeled amino acids liberated from aminoacyl-tRNA. ^{14}C-labeled amino acids were prepared from suppressor tRNA which was incubated with [^{14}C]serine, ATP, SerRS, tRNA kinase, H_2Se and enzyme C. Analyses were performed on silica gel G with a solvent of phenol/water (3:1). Arrows 1–4 denote positions of authentic Ps, Ser, Ala and SeCys, respectively.

incubated with H_2Se solution and enzyme C, is shown in fig.3. Fig.3A depicts the pattern of the complete system. The fraction of the casein peak in the figure contained 22 ng Se/mg casein. This value remained unchanged after incubation for 24 h. The amount of Se was found to be 6 mmol/mol casein (casein contains 9 mol phosphoserine). From these results, it was concluded that none of the phosphoserine residues on casein were converted to SeCys, but restricted phosphoserine residues on one species in some casein species should be catalyzed. The structure near the special residues on casein might resemble that surrounding Ps on Ps-GSHPx. Fig.3B illustrates the result when enzyme C was omitted, fig.3C showing the case in which glutathione reductase was absent. Fig.3D shows the case for passage of the fraction through the DEAE-cellulose column, instead of enzyme C. Fig.3E shows the result with enzyme A. Thus, the enzyme converting Ps-protein was found in the crude fraction (enzyme A) and was partially purified to enzyme C by ultracentrifugation and chromatography on DEAE-cellulose (activity bound on DEAE-cellulose). Further purification of this activity is planned for the future. Fig.3F shows the result for casein treated with alkaline phosphatase. The casein peak in fig.3B–D,F contained no Se. These results indicated that enzyme C, glutathione reductase and Ps on casein were essential for production of Se-protein. Fig.3G shows the result for omission of casein, indicating that some enzymes in the reaction mixture, such as enzyme C and glutathione reductase, do not contain Se. Fig.3H shows the case for albumin instead of casein, albumin not undergoing the change to Se-albumin. Thus, we considered that the reaction with enzyme C was as follows:



In order to confirm the presence of SeCys in Se-casein, the main casein peak in fig.3A was modified with iodoacetic acid and hydrolyzed. The hydrolyzate was analyzed by TLC, giving the

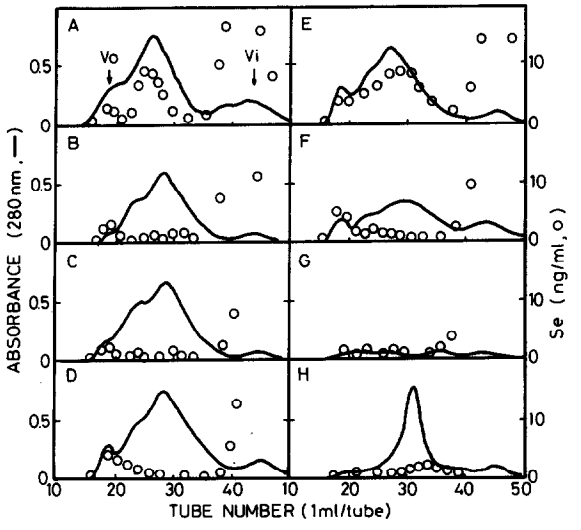


Fig.3. Chromatographic pattern of Se-casein on Sephacryl S-200. (——) Absorbance at 280 nm; (○) amount of Se. (A) Complete; (B) enzyme C omitted; (C) glutathione reductase omitted; (D) protein passed through the DEAE-cellulose column instead of enzyme C; (E) enzyme A instead of enzyme C; (F) casein treated with phosphatase; (G) casein omitted; (H) albumin instead of casein in A.

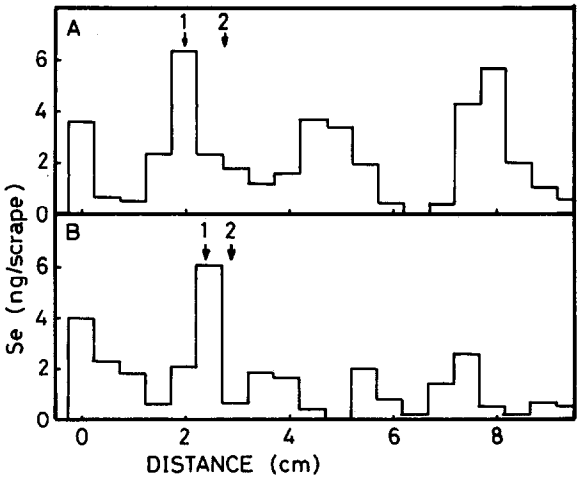


Fig.4. Analysis of hydrolyzates of CM-casein containing Se on silica gel G with solvent A (phenol/water, 3:1) and solvent B (*n*-butanol/acetic acid/water, 4:1:1). Arrows 1 and 2 indicate CM-SeCys and Ser, respectively.

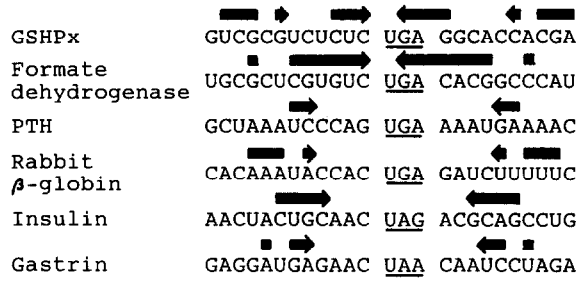


Fig.5. Comparison of secondary structures of termination regions of some mRNAs. Termination codons are underlined. 5′- and 3′-base-pairing regions are indicated by arrows.

results shown in fig.4. CM-SeCys was found at a position identical with that of authentic CM-SeCys in both solvent systems. CM-SeCys amounted to approx. 20–30% of total Se in the hydrolyzate. The results in fig.4 also show the presence of unidentified Se compounds in the hydrolyzate. It is not clear whether these unknown compounds were present in Se-casein or produced from SeCys during hydrolysis. Nevertheless, these results showed that Ps on phosphoprotein was converted to SeCys on the protein by some active enzymes. It is suggested that the phosphoprotein of GSHPx, in which Ps is incorporated at the position of the UGA codon through Ps-tRNA, is converted by some enzymes to mature GSHPx containing SeCys through a post-translational mechanism as are hydroxyproline and methyllysine [18]. Finally, we drew the conclusion that natural opal suppressor tRNA plays an important role in synthesizing GSHPx.

4. DISCUSSION

In formate dehydrogenase mRNA, it was shown that the 3′-flanking region of the UGA codon for SeCys could base-pair with the 5′-flanking region, this base-pair giving rise to the context effect for suppressor tRNA to read the UGA termination codon [13]. Fig.5 shows some sequences of mRNAs near the termination codons. GSHPx and formate dehydrogenase have some base pairs between the 5′- and 3′-flanking regions of the UGA codon. This base-pair has been found on mRNAs of human insulin and β-globin but good matching has not been observed for human PTH and gastrin. Thus, this base-pair is one of the specific points of mRNA of GSHPx and formate dehydrogenase. Another specific base-pair was between GA of the UGA codon and UC of the 5′-flanking region of UGA on the mRNAs of GSHPx and formate dehydrogenase. This base-pair was not found on the other mRNAs shown in fig.5. By this pairing, the release factor cannot recognize UGA but a tRNA specific to UGA may attack the base-pair and interact with UGA. The base-pair between the 5′- and 3′-flanking bases of UGA may play a role in stabilizing the pairing between GA of UGA and UC. Comparison between the regions near the UGA of mammals and of *E. coli* is interesting in that those regions near UGA, corresponding to SeCys, are able to compose the common structure of base-pairing.

The results in fig.1A,B do not coincide with those of Hawkes and Tappel [3,4] who showed that one species of $tRNA^{Cys}$ isoacceptors accepted SeCys. However, they did not demonstrate isolation of the tRNA specific to SeCys or SeCys-tRNA synthetase. In addition, they did not show that the tRNA containing Se corresponded to UGA or other codons. They found free SeCys in cytosol but the amount thereof was one half of that of SeCys-tRNA. Under normal conditions for protein synthesizing systems, amino acids are present at a concentration of about 20–50-fold that of the corresponding tRNAs. Therefore, the amount of free SeCys given in the results of Hawkes and Tappel is low. We determined the concentration of SeCys in rabbit reticulocytes to be lower than 2 nM. This value is about 0.01% of that of other normal amino acids. It is difficult to conceive the SeCys-tRNA synthesizing system with such a low concentration of SeCys. The chromatographic pattern obtained for Se-tRNA by Hawkes and Tappel is similar to that of tRNA containing minor Se nucleotides [19,20].

In fig.2, analyses of amino acids on aminoacyl-tRNA showed that part of the Ser-tRNA was converted to Ala-tRNA. This conversion proceeded in the presence of $tRNA^{Ser}$, SerRS and H_2Se solution, over a short period of time. This reaction was also observed with $tRNA^{Ser}_{IGA}$ as the major isoacceptor. This result was very interesting, however we considered that this reaction would proceed only in vitro, since, if this conversion proceeded in vivo, many unusual proteins, with Ala at the site of Ser, might be produced. Some organs possess SeCys

lyase which converts SeCys to Ala and H_2Se [21]. The same result for production of Ala-tRNA was obtained with an extract from testes, in which SeCys lyase is present in low amounts. This result indicated that Ala-tRNA was not converted from SeCys-tRNA. The reaction from Ser-tRNA to Ala-tRNA may proceed through dehydro-Ala-tRNA.

Ps is a high-energy compound [22], therefore conversion of Ps-tRNA or Ps-protein to SeCys-tRNA or SeCys-protein is easy. We were unable to conclude whether GSHPx was synthesized via a co- or post-translational mechanism. In the future, we shall attempt to clarify the mechanism using GSHPx mRNA and opal suppressor tRNA. We do not believe that the UGA codon changes to another codon after transcription, as in the case of apoB protein [23]. Other investigators have reported the absence of the GSHPx apo-enzyme in Se-deficient erythrocytes, using a monoclonal antibody specific to regions not relating to the active site of GSHPx [24]. They discussed the possibility that abnormal proteins such as an apo-enzyme may be unstable and decompose. Meanwhile, the presence of the precursor (28 kDa) of GSHPx (21 kDa) in the in vitro protein synthesizing system using GSHPx mRNA has been reported [25]. The precursor might be the apo-enzyme of GSHPx containing Ps. It was also considered that the synthesis of apo-enzyme and suppressor serine tRNA was regulated by the presence of Se, as in the relationship of metallothionein promoter and Zn.

This study suggests that natural opal suppressor tRNA must concern the synthesis of GSHPx. However, other unknown suppressors may be present in the cytosol. One species of $tRNA^{Trp}$ corresponds to UGA and some readthrough protein of β-globin has been produced [26], but in the case of SeCys, it is difficult to conceive the exchange of indole with Se. Some problems remain to be resolved. We have examined the conversion of Ps-tRNA to SeCys-tRNA or Ps-protein to SeCys-protein using H_2Se solution and some crude enzymes. We have described here that H_2Se in the solution might react with Ps-tRNA or casein, but the solution for producing H_2Se contains other Se compounds such as glutathione selenopersulfide (GSSeH) which must attack Ps-tRNA or casein [16]. At present, the metabolism of organic Se compounds from SeO_2 in the human body has not been clarified even though SeCys is present in the active center of GSHPx.

Acknowledgement: The authors thank Professor Kenji Soda of Kyoto University for the generous gift of L-selenocystine.

REFERENCES

[1] Forstrom, J.W., Zakowski, J.J. and Tappel, A.L. (1978) Biochemistry 17, 2639–2644.
[2] Stadtman, T.C. (1980) Annu. Rev. Biochem. 49, 93–110.
[3] Hawkes, W.C., Lyons, D.E. and Tappel, A.L. (1982) Biochim. Biophys. Acta 699, 183–191.
[4] Hawkes, W.C. and Tappel, A.L. (1983) Biochim. Biophys. Acta 739, 225–234.
[5] Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M. and Böck, A. (1988) Nature 331, 723–725.
[6] Sunde, R.A. and Evenson, J.K. (1985) Fed. Proc. 44, 1669.
[7] Sunde, R.A. and Evenson, J.K. (1987) J. Biol. Chem. 262, 933–937.
[8] Chambers, I., Frampton, J., Goldfard, P., Affara, N., McBain, W. and Harrison, P.R. (1986) EMBO J. 5, 1221–1227.
[9] Hatfield, D. (1985) Trends Biochem. Sci. 10, 201–204.
[10] Mizutani, T. and Hashimoto, A. (1984) FEBS Lett. 169, 319–322.
[11] Mizutani, T. and Tachibana, Y. (1986) FEBS Lett. 207, 162–166.
[12] Mizutani, T. and Hitaka, T. (1988) FEBS Lett. 226, 227–231.
[13] Zinoni, F., Birkmann, A., Stadtman, T.C. and Böck, A. (1986) Proc. Natl. Acad. Sci. USA 83, 4650–4654.
[14] Craigen, W.J., Cook, R.G., Tate, W.P. and Caskey, C.T. (1985) Proc. Natl. Acad. Sci. USA 82, 3616–3620.
[15] Mizutani, T., Narihara, T. and Hashimoto, A. (1984) Eur. J. Biochem. 143, 9–13.
[16] Ganther, H.E. (1971) Biochemistry 10, 4089–4098.
[17] Hall, R.J. and Gupta, P.L. (1969) Analyst 94, 292–299.
[18] Wold, F. (1981) Annu. Rev. Biochem. 50, 783–814.
[19] Ching, W.M. and Stadtman, T.C. (1982) Proc. Natl. Acad. Sci. USA 79, 374–377.
[20] Ching, W.H. (1984) Proc. Natl. Acad. Sci. USA 81, 3010–3013.
[21] Esaki, N., Nakamura, T., Tanaka, H. and Soda, K. (1982) J. Biol. Chem. 257, 4386–4391.
[22] Lipmann, F. (1984) Annu. Rev. Biochem. 53, 1–33.
[23] Powell, L.M., Wallis, S.C., Pearse, R.J., Edwards, Y.H., Knott, T.J. and Scott, J. (1987) Cell 50, 831–840.
[24] Takahashi, K. and Cohen, H.J. (1986) Blood 68, 640–645.
[25] Voigt, J.M. and Autor, A.P. (1983) Fed. Proc. 42, 806.
[26] Geller, A.I. and Rich, A. (1980) Science 283, 41–46.