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Some evidence of the enzymatic conversion of bovine suppressor phosphoseryl-tRNA to selenocysteyl-tRNA

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In order to clarify the mechanisms of selenocysteine incorporation into glutathione peroxidase, some evidence to show the in vitro conversion of phosphoseryl-tRNA to selenocysteyl-tRNA is reported. [^{3}H]Phosphoseryl-tRNA was incubated in a reaction mixture composed of SeO_2, glutathione and NADPH in the presence of selenium-transferase partially purified. Analyses of amino acids on the product tRNA showed that a part (4%) of [^{3}H]phosphoseryl-tRNA was changed to [^{3}H]selenocysteyl-tRNA. The conversion from seryl-tRNA^{su} or major seryl-tRNA$_{IGA}$ was not found. Selenium-transferase was essential for the conversion. [^{3}H]Selenocysteine, liberated from the tRNA, was modified with iodoacetic acid. The product was confirmed to be carboxymethyl-selenocysteine by two-dimensional TLC. Selenocysteyl-tRNA^{su} should be used to synthesize glutathione peroxidase by co-translational mechanisms.

tRNA, suppressor; Glutathione peroxidase; Selenocysteine; Opal nonsense codon; Phosphoseryl-tRNA

1. INTRODUCTION

Selenium is a trace and essential element for animals. Selenium was found as selenocysteine (SeCys) in the active site of glutathione peroxidase (GHSPx). GSHPx plays an important role in the detoxification of hydrogen peroxides, organic hydroperoxides and lipid peroxides. SeCys corresponds to the opal nonsense codon UGA on the murine and human mRNA of GSHPx [1,2]. In order to understand the mechanisms of incorporation of SeCys into GSHPx, the experiment with a perfused rat liver showed that the carbon source of the SeCys in GSHPx came from serine by specific labelling of SeCys with [^{14}C]Ser [3]. Another enzyme containing SeCys is formate dehydrogenase (FDH) in *Escherichia coli*. SeCys in FDH was incorporated through Ser-tRNA by co-translational mechanisms [4,5]. The synthesis of SeCys from selenite has not been clarified in prokaryotes and eukaryotes.

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Meanwhile, in mammals, natural suppressor tRNA corresponding to UGA was found about two decades ago [6,7]. This tRNA accepts serine and the read-through protein of β-globin, having UGA termination codon, was synthesized [8]. This suppressor tRNA was phosphorylated to phosphoseryl(Ps)-tRNA^{su} by tRNA kinase [9] and the incorporation of Ps into the read-through protein of β-globin was found [10]. The role of this tRNA^{su} is not clear. The possibility of participation of Ps-tRNA in phosphoserine aminotransferase catalysis was excluded [11]. Natural tRNA^{su} did not participate in the regulation of the release reaction of RF [12], which has a stronger affinity to the nonsense codon UGA (K_a for Ps-tRNA:UGA, 8×10^3 M^{-1}; K_a for RF:UGA, 1.26×10^6 M^{-1}). The possibility remains that this natural suppressor tRNA participates in the incorporation of SeCys into GSHPx. In the previous paper [13], we studied the mechanisms of incorporation of SeCys into GSHPx through Ps-tRNA and suggested the conversion of Ps-tRNA to SeCys-tRNA. However, we could not show clear evidence of conversion of Ps-tRNA to SeCys-tRNA. In this paper I show some evidence of the

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in vitro conversion of [^{3}H]Ps-tRNA to SeCys-tRNA.

2. MATERIALS AND METHODS

Bovine suppressor tRNA, bovine SerRS and murine tRNA kinase were prepared according to [9,14]. [^{3}H]Ser-tRNA was prepared with [^{3}H]serine (37 Ci/mmol; Amersham) and purified on Sephacryl S-200 [13]. Then [^{3}H]Ser-tRNA was phosphorylated with cold ATP and tRNA kinase. Ps-tRNA was purified on Sephacryl S-200 (1.1 × 40 cm) in 0.15 M NaCl–10 mM acetate buffer at pH 4.6 [9]. In this preparation, Ps-tRNA was 5% of the total [^{3}H]Ser-tRNA by analyses of [^{3}H]amino acids liberated from the tRNA on AG-1 columns (95% of total ^{3}H were passed through the column and 5% were eluted with 0.1 M NaCl–0.01 M HCl). [^{3}H]Ps-tRNA was collected by ethanol precipitation. [^{3}H]Ps-tRNA was dissolved in 0.1 ml of 0.1 M Hepes at pH 6.5 and then the solution was mixed with 0.1 ml of the 'H_2Se' solution [15], which was composed of 0.1 mM selenite, 16 mM GSH, 0.4 mM NADPH, 40 mM mercaptoethanol, 20 mM Hepes (pH 6.5), and 1 μg yeast glutathione reductase (Boehringer Mannheim), previously incubated for 30 min at 30°C. Then Se-transferase preparation (20 μl, 0.1 mg/ml) was added to the mixture of Ps-tRNA and the 'H_2Se' solution. The final concentrations of Se and GSH in the reaction mixture are close to those in blood. This solution was incubated for 30 min at 30°C and then acidified by the addition of 0.2 ml of 1 M acetate (pH 4.6). The tRNA^{su} in the mixture was precipitated with ethanol and dried. Then tRNA^{su} was dissolved in 20 μl of 20 mM Tris-5 mM mercaptoethanol at pH 9, to liberate amino acids from aminoacyl-tRNA, and the solution was incubated at 30°C for 1 h. tRNA in the mixture was removed by precipitation with ethanol and the supernatant was used for analysis. [^{3}H]Amino acid was analyzed by TLC on silica gel G in phenol:water (3:1) or *n*-butanol:acetic acid:water (4:1:1). Authentic SeCys, CM-SeCys, serine, leucine and glutamic acid were co-chromatographed. After developing, the plates were dried and colored with 0.3% ninhydrin solution in acetone. Then silica on plates was scraped and the radioactivity was measured. One part of silica, scraped from the position of SeCys, was suspended in 20 mM iodoacetic acid at pH 7 and incubated overnight at 20°C. The supernatant of the reaction mixture was analyzed by TLC. The amount of Se was determined according to [13].

Se-transferase from murine liver was partially purified on DEAE-cellulose and Sephacryl S-300. In order to know the fraction containing Se-transferase activity, an eluate from the column was assayed according to the previous report [13]. [^{3}H]SeCys on the product tRNA was chromatographed on silica gel and ^{3}H radioactivity in the spot of cold SeCys was counted. The activity profile of Se-transferase on the chromatographic patterns using Ps-tRNA as substrate was consistent with the pattern of casein. Sometimes, kinetic parameters of Se-transferase were obtained from the amount of Se in the tRNA or casein fraction on Sephacryl S-200.

3. RESULTS AND DISCUSSION

[^{3}H]Ps-tRNA was incubated with the 'H_2Se' solution in the presence of Se-transferase. The results of analyses of the product are shown in fig.1. Fig.1a shows the result with use of an eluate from DEAE-cellulose as Se-transferase. Arrow 1 in fig.1a shows the position of serine. Arrow 2 shows the position of authentic cold SeCys co-chromatographed. The peak in front of arrow 2 corresponds to aliphatic amino acids, such as leucine and methionine, but I have not identified this spot. Some ^{3}H radioactivity was clearly found at the position coincident with SeCys in fig.1a. The amount of ^{3}H radioactivity in the spot of SeCys was 0.2% of the total ^{3}H radioactivity applied on the TLC plate. This value indicates that 4% of Ps-tRNA was converted to SeCys, because Ps-tRNA was 5% of the total [^{3}H]Ser-tRNA as described in section 2. This value, 4% of the product, is the standard level of many enzyme reactions and meaningful. It is difficult to get more SeCys-tRNA in vitro, because Ps-tRNA is labile at neutral pH and hydrolyzed during long-time incubation. Therefore, the reaction was carried out by a restricted one set condition, such as at pH 6.5, 30°C and for 30 min as described in section 2. A more effective reaction from seryl-tRNA to SeCys-tRNA through Ps-tRNA should be present in vivo, especially in the organelle synthesizing GSHPx such as reticulocytes. In our previous report [13], a small amount (0.016%) of Ps on casein was converted to SeCys, but this value was much lower than the above value (4%) of SeCys-tRNA. Therefore, we concluded that SeCys binding on tRNA^{su} should be incorporated into GSHPx by co-translational mechanisms. In vertebrates, co-translational incorporation should be a major pathway.

Ser-tRNA^{su} is used in fig.1b instead of Ps-tRNA as a substrate in fig.1a. We could not find any conversion of Ser-tRNA^{su} to SeCys-tRNA, as shown in fig.1b. The results of fig.1a and b showed that SeCys-tRNA was not synthesized from Ser-tRNA^{su} but Ps-tRNA. Only Ps-tRNA is an effective substrate for Se-transferase. Thus, SeCys-tRNA is synthesized in eukaryotes as follows:

$$\text{tRNA}^{su} \xrightarrow[\text{SerRS}]{} \text{Ser-tRNA}^{su} \xrightarrow[\text{tRNA kinase}]{}$$

$$\text{Ps-tRNA}^{su} \xrightarrow[\text{Se-transferase}]{} \text{SeCys-tRNA}$$

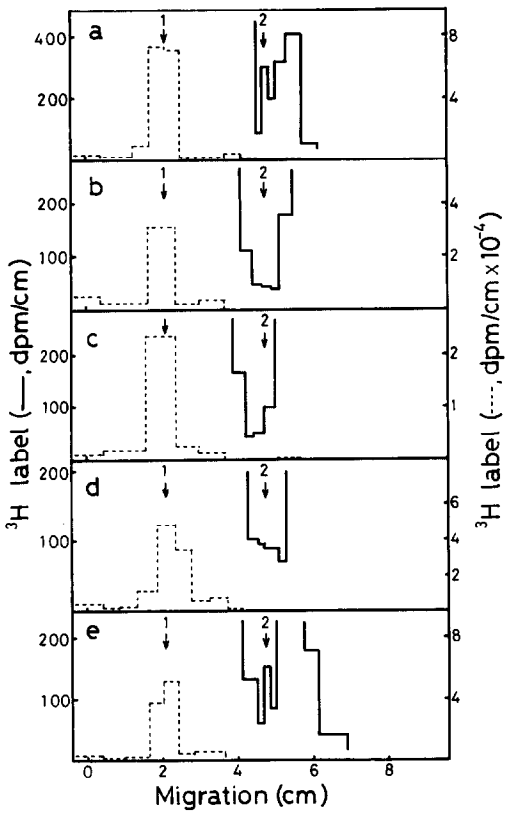

Fig.1. Analyses of [^{3}H]amino acids liberated from aminoacyl-tRNA^{su} by TLC on silica gel G in phenol:water (3:1). Authentic SeCys and serine are indicated by arrows (1, serine; 2, SeCys). Silica on plates was scraped and the radioactivity was measured. (a) The result of the complete system of [^{3}H]Ps-tRNA reacted with an eluate (Se-transferase) from a DEAE-cellulose column on which the supernatant at 105000 × *g* of murine liver extracts was chromatographed. (b) [^{3}H]Ser-tRNA^{su} was used instead of Ps-tRNA in a. (c) Major [^{3}H]Ser-tRNA$_{IGA}$ was used instead of Ps-tRNA in a. (d) An enzyme preparation (Se-transferase in a) was omitted. (e) Se-transferase further purified on Sephacryl S-300 was used instead of the DEAE-cellulose fraction in a. [^{3}H]Ser used was a product of Amersham (37 Ci/mmol).

Fig.1c shows the result of [^{3}H]Ser-tRNA$_{IGA}$ (the major isoacceptor of serine tRNA) instead of Ps-tRNA^{su} in fig.1a. Major Ser-tRNA was also not converted to SeCys-tRNA. In mammals, three serine tRNAs (tRNA$^{Ser}_{IGA}$, tRNA$^{Ser}_{GCU}$, and tRNA$^{Ser}_{NCA}$) were recognized with the same affinity (K_m) and velocity (V_{max}) by bovine SerRS [14,16]. Therefore, we pointed out that the anticodon sequence of serine tRNAs should not be involved in the recognition mechanisms between the tRNAs and SerRS [14], even though MetRS recognizes the anticodon on tRNA^{Met} [17].

Fig.1d shows the case where Se-transferase is omitted. The protein fraction containing Se-transferase is essential to the conversion of Ps-tRNA to SeCys-tRNA. This activity was eluted at 0.05 M KCl by gradient elution from a DEAE-cellulose column as shown in fig.2a. The passed-through fraction DEAE-cellulose did not contain any activity. The active fraction from DEAE-cellulose was further purified on Sephacryl S-300 and the activity was found in the passed-through fraction as shown in fig.2b. This result suggests that Se-transferase was high-molecular mass. These elution properties of Se-transferase from DEAE-cellulose and Sephacryl S-300 were different from those of SerRS [14] and tRNA kinase [9]. Fig.1e shows the result with the fraction (fig.2b) obtained from Sephacryl S-300 as Se-transferase, instead of the DEAE-cellulose frac-

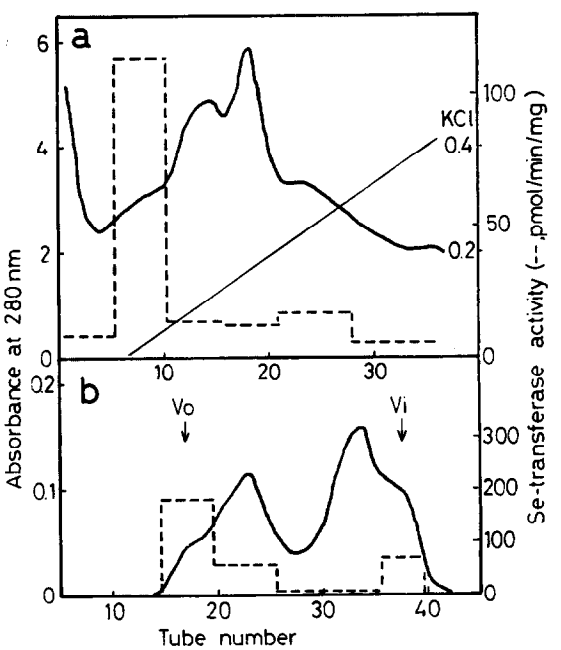

Fig.2. Purification of Se-transferase. (a) An elution pattern on a DEAE-cellulose column (40 × 1.6 cm) of a supernatant (specific activity 8 pmol/mg per min) at 105000 × *g* of a murine liver extract (0.6 g protein). Elution was carried out with a linear gradient (0–0.5 M KCl, total volume 200 ml) and a fraction volume was 5 ml. (b) An elution pattern on a Sephacryl S-300 column (40 × 1.6 cm) of the active fraction in a. Elution buffer was 10 mM Tris-HCl, 10 mM mercaptoethanol, 10 mM $MgCl_2$ and 5% glycerol. A fraction volume was 2 ml.

tion in fig.1a. The activity was also found in the supernatant at 0.6 saturation of ammonium sulfate by fractional precipitation. Thus, the activity to convert Ps-tRNA to SeCys-tRNA was found in the supernatant at 105000 × *g* of liver extract and partially purified as shown in fig.2. The tentative K_m values for Se compounds and Ps compounds were 20 μM and 0.2 μM, respectively.

Fig.3 shows the results of analyses of SeCys in the complete system on TLC in another solvent, *n*-butanol:acetic acid:water. ^{3}H radioactivity was found at the position of authentic SeCys with a similar yield, 0.25% of total ^{3}H, to that in fig.1a. Then, SeCys was further ascertained after modification with iodoacetic acid. SeCys, obtained by scraping the position of SeCys on TLC in solvent phenol:water (fig.1a), was carboxymethylated with iodoacetic acid and then analyzed by two-dimensional TLC as shown in fig.4. The numbers in the squares of the plate in fig.4 show the amount of ^{3}H radioactivity in scrapes (cpm). The values of blank level radioactivity on the plate were omitted in fig.4. Most of the radioactivity spotted on the plate was clearly found at the position of authentic CM-SeCys by two-dimensional TLC (74% recovery). No radioactivity was found at the position of SeCys of the raw material. Thus, I confirmed that Ps-tRNA is converted to SeCys-tRNA by a Se-transferase. SeCys on this SeCys-tRNA should be incorporated into GSHPx.

I used the 'H_2Se' solution to synthesize SeCys-tRNA. Fig.5 shows the results of analyses of the 'H_2Se' solution on TLC in the solvent *n*-butanol:acetic acid:water. The upper part of fig.5 shows the TLC plate colored with ninhydrin. The spot near the origin of the complete system was not found in the absence of SeO_2. This position coincided with the position of oxidized-glutathione. Therefore, glutathione was oxidized with SeO_2 to become oxidized glutathione. The lower part of fig.5 shows the amount of Se on TLC plate. One major spot is similar to the position of oxidized-glutathione and should be glutathione selenotrisulfide (GSSeSG) according to the literature [15]. Another major spot of Se on TLC was found near the position of H_2O_2 and should be H_2Se. The new 'H_2Se' solution contained these two Se compounds but the old one after storage for one week or longer contained SeO_2, GSSeSG and H_2Se. The extracts from these two spots

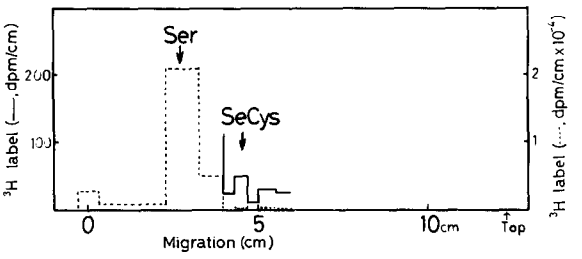

Fig.3. Analyses of [^{3}H]amino acid liberated from SeCys-tRNA by TLC on silica gel G in *n*-butanol:acetic acid:water (4:1:1).

(GSSeSG and H_2SE) were active as substrates to synthesize SeCys-tRNA. GSSeSG was labile and immediately changed to carboxymethyl-glutathioneselenopersulfide by the treatment with iodoacetic acid.

This report showed that natural suppressor tRNA has a function to incorporate SeCys into GSHPx through Ps-tRNA. The function of tRNA kinase is also essential to synthesize Ps-tRNA which is converted to SeCys-tRNA by Se-transferase. The mechanisms of specific recognition of UGA by natural suppressor tRNA were elucidated by the context effect, which introduced by base-pairing between GA of UGA for SeCys and UC of the 5′-flanking region of UGA on the

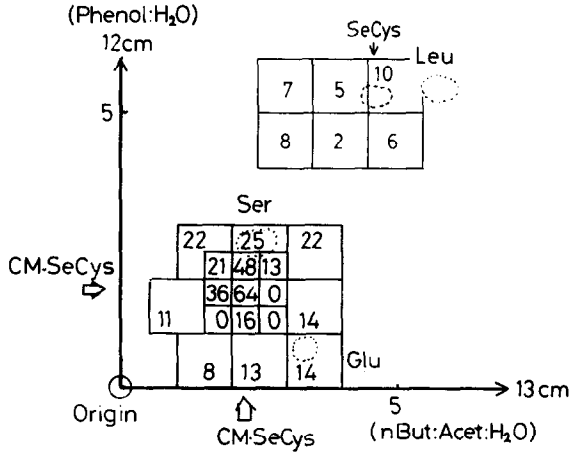

Fig.4. Analyses of carboxymethyl [^{3}H]SeCys by two-dimensional TLC on silica gel G. First dimension (developed 13 cm), *n*-butanol:acetic acid:water (4:1:1); 2nd dimension (developed 12 cm), phenol:water (3:1). Dotted circles indicate the position of standard amino acids. Arrows indicate the position of CM-SeCys. The numbers in the squares indicate ^{3}H radioactivity in those scrapes.

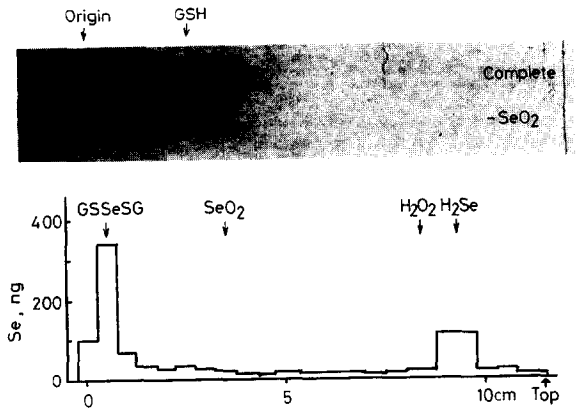

Fig.5. Analyses of the 'H_2Se' solution by TLC on silica gel G in *n*-butanol:acetic acid:water (4:1:1). The upper part shows the result of colored plate with ninhydrin; the lower part shows the amount of Se in scrapes in upper plate. The positions of authentic standard are indicated in the figure.

mRNAs of GSHPx and FDH [1,18]. The amino acids corresponding to XUC are Ile, Leu, Val and Phe. As a result of research of termination codons of some hundreds of structure genes, in 64 genes having the UGA termination codon, 3 genes have XUC codon at the 5′-flanking regions of UGA [19–21]. Thus, protein genes having the base-pair between GA of UGA and UC of the 5′-flanking region of UGA are not the rare case but are present at the mean level; therefore, it is difficult to understand the context effect from the base-pair neighbor, the UGA codon. Another problem to resolve is the discrimination among Ser-$tRNA^{su}$, Ps-$tRNA^{su}$ and SeCys-$tRNA^{su}$. These have the same tRNA structure but different amino acids. It is difficult to assume that the discrimination is carried out at the levels of protein synthesis on ribosomes or the interaction with eEF1α. We therefore assume that the incorporation of SeCys into GSHPx depends upon the ratio of those aminoacyl residues on $tRNA^{su}$. The in vivo ratio of those tRNAs in cytosol must be confirmed (in vivo, the presence of SeCys-tRNA should be dominant).

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