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Trace 5-Methylaminomethyl-2-selenouridine in bovine tRNA and the selenouridine synthase activity in bovine liver

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Abstract

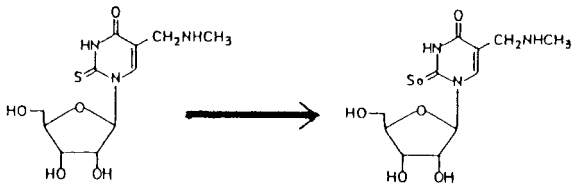
We measured the amount of Se in bovine liver tRNA. tRNA was chromatographed on a BD-cellulose column and Se-rich tRNA was eluted from the column in front of a main tRNA peak. There was 0.3 mmol Se/mol of tRNA and this level is about one tenth that of *Escherichia coli* tRNA. This suggests the presence of an enzyme that modifies tRNA with Se in bovine liver. We isolated the activity of this enzyme (selenouridine synthase) by chromatography of bovine liver extracts on a DEAE-cellulose column. ATP and selenophosphate synthetase, as well as selenouridine synthase and tRNA, were necessary for the reaction. ^{75}Se was used to label the reaction products, which were analyzed by TLC after digestion with ribonuclease T_2. The position of the ^{75}Se-nucleotide on a TLC plate was identical to that of the Se-nucleotide, 5-methylaminomethyl-2-seleno-Up, prepared from ^{75}Se-tRNA in *E. coli*.

Abbreviations: SePS – selenophosphate synthetase; mnm^5Se^2U – 5-methylaminomethyl-2-selenouridine; mnm^5Se^2Up – the nucleotide; BD-cellulose – benzoylated-DEAE-cellulose.

Introduction

Some modified uridines, such as 5-hydroxyuridine and mo^5U, at the wobble position of the anticodon of tRNA, can form base pairs with G and U by non-Watson-Crick base pairing, as well as with A [1, 2]. Another modified base ac^4U can only base-pair with G. Meanwhile, 2-thiouridine derivatives, such as mnm^5s^2U, mcm^5s^2U and $cmnm^5s^2U$, can only form base pairs with A and G. This base-pair specificity prevents translation errors. Recognition of the correct codon recognition may be facilitated by the intrinsic conformational rigidity of the mnm^5U residue in the presence of the 5′-phosphate and 2′-methyl groups [3].

$tRNA^{Glu}$ and $tRNA^{Lys}$ in *Escherichia coli* or in *Methanococcus vannielii* contain 5-methylaminomethyl-2-selenouridine (mnm^5Se^2U) [4, 5] and this nucleoside is produced by the conversion from 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), as follows:



Se-nucleosides in the anticodon strengthen the interaction with the codon [6]. It has also been reported that there is an enzyme for modification of mnm^5s^2U to mnm^5Se^2U, know as selenouridine synthase in *E. coli* [7].

Selenium is an essential trace element in animals [8]. Selenium deficiency causes human diseases including Keshan disease, which is endemic in China [9]. Keshan disease can be prevented by administration of selenite. Selenium is found in the active sites of glutathione peroxidase and type-I iodothyronine 5′-deiodinase as selenocysteine. Selenocysteine is produced from the seryl-residue on tRNA (Seryl-$tRNA^{Sec}$) by selenocysteine synthase [10]. In this reaction, it is believed that the Se-donor is selenophos-

phate, produced from hydrogen selenide and ATP by selenophosphate synthetase [11]. It is possible that the same Se-donor, selenophosphate, is used for base modification of tRNA by a selenium transfer reaction. In this report, we show the presence of Se-nucleoside in tRNA prepared from bovine liver and the the presence of a selenouridine synthase in bovine liver cytosol [12].

Experimental

tRNA. tRNA was isolated from bovine liver by a standard phenol extraction method, according to a previous paper [13]. One gram of tRNA was obtained from approximately one kg of fresh bovine liver. Two grams of tRNA (32 000 A_{260} units) were applied to a benzoylated-DEAE(BD)-cellulose column (5×30 cm) and chromatographed using a linear gradient of 0.4 M to 1.5 M NaCl in 10 mM acetate at pH 4.6 and 10 mM $MgCl_2$ (total volume, 4 l) [13]. The tRNA eluates from the BD-cellulose column were precipitated by addition of ethanol and the precipitates were collected by centrifugation.

Preparation of enzymes. Enzymes were prepared from bovine liver extracts, which were centrifuged for 1 h at 150 000 $\times$ *g* after which the supernatant was chromatographed on DEAE-cellulose [14]. Selenophosphate synthetase activity eluted at 0.2 M KCl and was further purified by fractional precipitation with ammonium sulfate, gel filtration on Sephacryl S-300 and heparin-Toyopearl. The seryl-tRNA synthetase used was purified as described previously [14].

Enzyme assay. Serylation activity of tRNA was measured by a previous method [14] as follows. A 50 μl mixture containing tRNA, [^{14}C]Ser, 5 mM ATP, and seryl-tRNA synthetase in 0.2 M Hepes at pH 7.6, 20 mM KCl and 20 mM $MgCl_2$ was incubated at 37°C for 30 min and then the mixture was spotted onto a filter paper. The paper was dipped in cold 10% TCA to cause tRNA precipitation and then washed with cold 0.2 M HCl. After drying, the radioactivity on the filter paper was quantitated with a liquid scintillation counter, and the [^{14}C]seryl-tRNA level was calculated.

Selenouridine synthase activity was measured as follows. The assay mixture (50 μl) was composed of a DEAE-cellulose fraction (20 μl, 0.16 mg protein), partially purified selenophosphate synthetase (10 μl), $H_2{}^{75}Se$ (2 μl, 20 Ci/mol), 5 mM ATP, tRNA (25 μg) in 0.2 M Hepes-20 mM KCl-20 mM $mgCl_2$. This mixture was incubated for 30 min at 30 °C and then mixed with phenol. The tRNA in the supernatant was precipitated by addition of 2 volumes of ethanol and the precipitate was dissolved in 5 μl water and spotted onto a filter paper, which was then washed with cold 10% TCA. The radioactivity on the filter paper was measured with the Fuji Bioimage Analyzer BAS 2500. The tRNA saturation concentration was 1 A_{260} unit/ml and the protein saturation concentration was 3 mg/ml.

Se levels in tRNA. The Se level in tRNA was measured by fluorometry with diaminonaphthalene [15], as follows. tRNA (5 mg) was hydrolyzed in 60% $HClO_4$-40% H_2SO_4. After the conversion of SeO_3 to SeO_2 by the addition of cHCl, the mixture was neutralized with cNH_4OH and a stabilizing solution containing NH_2OH-EDTA. Diaminonaphthalene in 0.2N HCl was added and incubated at 50 °C. The diaminonaphthalene-selenol complex was extracted with cyclohexane and the concentration of the diaminonaphthalene-selenol complex in cyclohexane was measured using a fluoroscence spectrophotometer (Hitachi F2000) at an excitation wavelength of 360 nm and an emission wavelength of 520 nm.

Preparation of authentic Se-nucleotide from E. coli. In order to characterize the Se-nucleotide produced by selenouridine synthase, we prepared modified [^{75}Se]nucleotide from *E. coli*, in which mnm^{5}Se^{2}Up is the major selenonucleotide [4]. Two strains of *E. coli* JM109 and MC1061 were cultured in 15 ml of LB medium containing 0.2 mCi/nmol [^{75}Se]selenite. The level of incorporation of ^{75}Se was higher in MC1061 so we used this strain to prepare Se-nucleotide. *E. coli* pellets were lysed with lysozyme and SDS, and nucleic acids were precipitated by addition of two volumes of ethanol. ^{75}Se-tRNA was fractionated on BD-cellulose. A peak of ^{75}Se appeared in the middle of the elution pattern [4]. tRNA containing ^{75}Se was collected and digested with ribonuclease T_2 (2 units) for overnight at 37 °C, and used as authentic mnm^{5}Se^{2}Up without further purification [16].

Analysis of Se-nucleotide. ^{75}Se-tRNA produced using bovine liver selenouridine synthase was hydrolyzed with ribonuclease T_2 by the procedures shown above. The hydrolyzate with ribonuclease T_2 was composed of nucleotides having 3′-phosphate and we analyzed ^{75}Se-hydrolyzate as nucleotides (not nucleosides). The hydrolyzate was analyzed by the

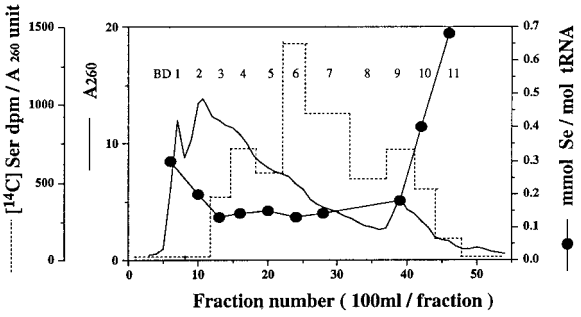


Figure 1. Elution pattern of bovine liver tRNA on BD-cellulose. Bovine tRNA (32 000 A_{260} units) was chromatographed on a column (5 × 30 cm). Experimental conditions are described in Experimental. The broken line represents Ser accepting activity. Closed circles indicate the Se-level. BD1-9 are tRNA fractions as described in text.

Kuchino′s method [17], on Avicel (Funakoshi) by secondary TLC (solvent for the first dimension, isolactic acid:0.5 M ammonia = 5 : 3, and the second dimension, isopropanol:cHCl:water = 75 : 15 : 15). ^{75}Se-nucleotides on the thin layer were detected by autoradiography using a Fuji Bioimage Analyzer BAS 2500.

Results

Se in bovine liver tRNA. Mammalian tRNA was prepared from bovine liver by the standard methods with phenol-SDS [13]. Active tRNA was chromatographed on BD-cellulose as shown in Figure 1. The Ser-accepting activity and the amount of Se in fractions eluted from the BD-cellulose column were measured by methods used in previous reports [13, 15]. Serine tRNAs in fractions 4, 6 and 9 in Figure 1 are $tRNA_{GCU}$, $tRNA_{IGA}$ and $tRNA_{UCA}$, respectively. Se concentrations were highest in the early eluting fractions and in the last fraction. The last fraction was contaminated, as it did not exhibit any amino acid accepting activity. The Se level in the early eluting fraction was 0.8 ng/mg of tRNA (0.3 mmol Se/mol of tRNA). The Se level in fractions 3–9 was 0.1 mmol/mol of tRNA. The Se level in fraction BD1 in Figure 1 was lower than the level of one tRNA species was fully modified with Se.

Selenouridine synthase activity. We hypothesized that bovine liver cytosol contains a selenouridine synthase, because of significant Se levels in the tRNA. We analyzed the selenouridine synthase activity in the bovine liver extract by centrifugation at 150 000 × *g*

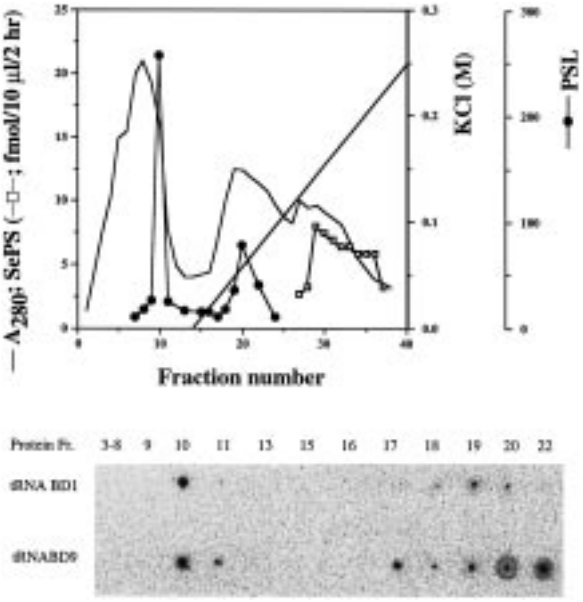


Figure 2. Selenouridine synthase activity of fractions of bovine liver extracts chromatographed on DEAE-cellulose. The upper part of the figure is an elution pattern of the bovine liver extract from a DEAE-cellulose column. Closed circles indicate selenouridine synthase activity in the fractions (radioactivity level of ^{75}Se bound to tRNA from fraction BD1 measured from the blots in this lower picture). Open squares indicate selenophosphate synthetase (SePS) activity in those fractions. The straight line indicates the concentration of KCl. PSL (arbitrary units of photosensitive luminescence by BAS 2500) indicates the level of ^{75}Se radioactivity. The lower figure is the result of dot blot analysis of ^{75}Se-tRNA modified by selenouridine synthase in the fractions from DEAE-cellulose. The radioactivity of each blot with BD1 was plotted in the upper chromatograohic pattern. The precipitate was washed with 10% cold TCA. Fraction 10 has activity when incubated with tRNA from fractions BD1 and BD9.

after which the supernatant was chromatographed on DEAE-cellulose. The elution pattern is shown in the upper part of Figure 2. We used the tRNA in fractions BD1 and BD9, as shown in Figure 1, as the Se-acceptor for the selenouridine synthase reaction. The tRNA and eluates, shown in Figure 2, were incubated as described in Experimental. The tRNA in this mixture was collected by ethanol precipitation. The precipitate was dissolved in water and spotted onto filter paper, and then washed in 10% cold TCA, the results of which are shown in the lower part of Figure 2. The radioactivity patterns of the tRNA from BD1 and BD9 were similar, because it was found that the protein fraction 10 enzymatically modified the tRNA in fractions BD1 and BD9 with selenium. This indicates that an enzyme in protein fraction 10 can react with not only BD1 which contains a high level of Se tRNA but

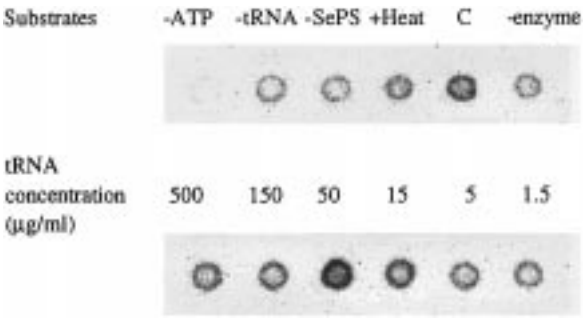


Figure 3. The reaction conditions of selenouridine synthase. The upper row shows the substrates necessary for selenouridine synthase activity. 'C' is the complete reaction mixture and contains 20 μl of selenophosphate synthetase (SePS), 1 μl of $H_2{}^{75}Se$ (14 μCi/ml; 7 mM), 5 μl of 50 mM ATP, 5 μl of tRNA (15 mg/ml), and 20 μl of enzyme. Captions in the upper row show the components (ATP, tRNA, SePS, or enzyme) omitted from the complete reaction solution. +Heat indicates the enzyme was heat-treated at 95 °C for 5 min. The lower row shows the optimum tRNA concentration. The lower row contains identical amounts of all the substrates except tRNA as in the upper columns.

also BD9 which contains a low level of Se-modified tRNA. Meanwhile, the difference between the patterns of the two tRNA fractions was appeared using protein fractions 20–22 in the lower part of Figure 2, this may come from [^{75}Se]SeCys-tRNA by BD9 and its enzymes in these fractions. Finally, we determined that there was selenouridine synthase activity in protein fraction 10.

Assay conditions of selenouridine synthase. Based on a scheme created by Leinfelder et al. [18], there was no discrimination between the selenouridine synthase and selenophosphate synthetase in *E. coli*., however we did not believe that was true for mammals. We isolated selenouridine synthase activity in protein fraction 10, as shown in the upper part of Figure 2. Next, we demonstrated that this activity is different from selenophosphate synthetase activity. The upper row of Figure 3 shows that the reaction proceeds in the presence of selenophosphate synthetase and ATP. In the absence of ATP, selenophosphate synthetase, or tRNA, the reaction does not proceed. The enzyme had weak activity after heat treatment indicated by +Heat in the figure. These results show that the base modification reaction needs both the activities of selenouridine synthase and selenophosphate synthetase, as well as ATP and tRNA. This also shows that the Se-donor of the selenouridine synthase reaction is selenophosphate. The lower row of Figure 3 shows the dose-response relationship of the tRNA. The maximum activity of

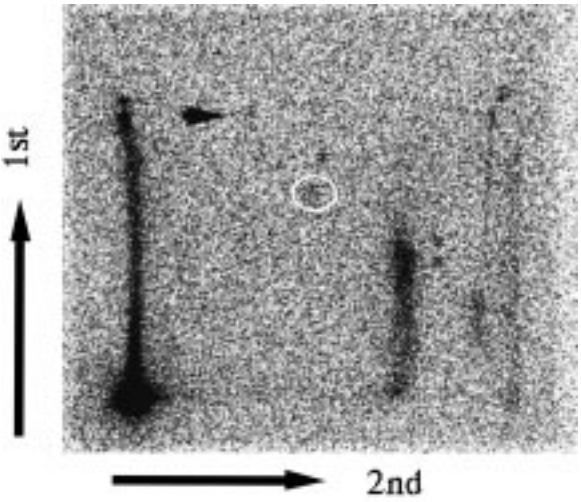


Figure 4. Analysis of ^{75}Se-nucleotide on silica gel G. The results of the analysis of the bovine ^{75}Se-tRNA modified by selenouridine synthase. Experimental conditions for TLC are described in Experimental. The white circle indicates the position of the major ^{75}Se-nucleotide. The arrow indicates the minor spot of ^{75}Se-nucleotide. * and ** indicate the positions of standard Cp and Up, respectively.

the modification enzyme appears at the concentration of 1 A_{260} unit of tRNA/ml (50 μg/ml). Selenophosphate synthetase used in this study was prepared from the fraction (fraction 29–36) eluted from a DEAE-cellulose column at a concentration of 0.2 M KCl, indicated in the upper part of Figure 2, meanwhile, the selenouridine synthase activity was found in the unbound and retarding fraction (fraction 10 in Figure 2, this fraction has a weak affinity to DEAE-cellulose) from the column as shown in the upper part of Figure 2. Thus, selenouridine synthase and selenophosphate synthetase activities were clearly separated on DEAE-cellulose.

Identification of Se-nucleotide as mnm^{5}Se^{2}Up. It has been reported that the tRNA in *E. coli* contains mnm^{5}Se^{2}Up [4]. We cultured *E. coli* in the presence of [^{75}Se]SeO_2 and prepared [^{75}Se]tRNA from *E. coli*. This ^{75}Se-tRNA was fractionated on BD-cellulose and the ^{75}Se-tRNA was eluted from the middle of the tRNA chromatographic pattern. The ^{75}Se-tRNA peak was concentated by ethanol precipitation, digested with ribonuclease T_2, and used as authentic mnm^{5}Se^{2}Up [19]. Bovine tRNA (BD1) was modified with ^{75}Se by selenouridine synthase in the presence of selenophosphate synthetase and ATP. ^{75}Se-tRNA was digested with ribonuclease T_2. The digest was analyzed by 2-dimensional TLC as shown in Figure 4.

The spot indicated by the white circle in the figure contained a major ^{75}Se-nucleotide. This Se-nucleotide was confirmed as mnm^{5}Se^{2}Up, because this position was identical to that of mnm^{5}Se^{2}Up, the authentic Se-nucleotide of *E. coli* (data not shown). This was supported by the relative position to that of Cp and Up. Because the position was near the position of mnm^{5}s^{2}Up shown by the Kuchino′s method [17] and the poistion of mnm^{5}s^{2}Up was near the position of Cp as shown by the single asterisk in the figure. Thus, we concluded the position of Se-nucleotide circled in the figure is mnm^{5}Se^{2}Up. Another minor ^{75}Se spot indicated by an arrow in Figure 4 is near the position of Ap and may be mSe^{2}t^{6}Ap [20]. Other smeared spots were impurities found in blank experiments and may be due to inorganic ^{75}Se such as selenite and/or selenide. These results show that the major Se-nucleotide in bovine liver is mnm^{5}Se^{2}Up, although the aminoacylated tRNA species remains unclear.

Discussion

In this work, we have demonstrated the presence of 0.3 mmol Se/mol of tRNA in bovine liver. This value is one-tenth the level of Se in *E. coli* tRNA [19], suggesting that only select tRNA species were modified. It is possible that Se-modified tRNA may play a role in regulation of translation, by the methods of that Se-nucleotides in the anticodon strengthen the interaction with the codon [6].

We have isolated selenouridine synthase activity, with properties similar to mnm-selenouridine synthase in *E. coli*, and demonstrated that selenophosphate was necessary as the Se-donor in this reaction. Therefore, the systematic name of this enzyme is selenophosphate: tRNA seleniumtransferase. Selenophosphate is also the Se-donor in selenocysteine synthesis of tRNA^{Sec} by conversion from Ser-tRNA^{Sec} to selenocysteyl-tRNA^{Sec} [10].

In the scheme in Figure 5, we illustrated how selenophosphate is used to synthesize mnm^{5}Se^{2}U by selenouridine synthase and selenocysteyl-tRNA by selenocysteine synthase. Se is a trace element and the level in our bodies is 0.2 ppm (2.5 nM). Most Se in our bodies binds to proteins and a part of unbund Se should be selenophosphate. And selenophosphate is estimated to be the level of pM in our bodies. We investigate the stability of selenophosphate in various conditions and those results will be published in elsewhere.

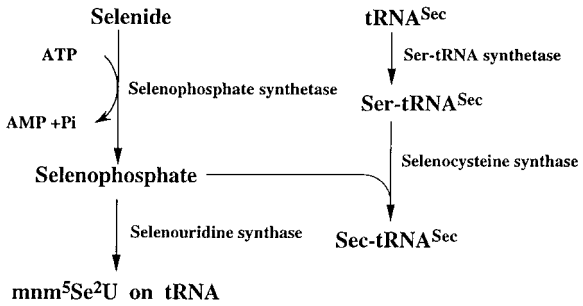


Figure 5. Scheme of Se incorporation into selenocysteine and Se-nucleotide in mammals.

The specific tRNA species that was modified is unclear. Generally, the early eluting fraction from BD-cellulose contains tRNA^{Met}, tRNA^{Glu}, tRNA^{Val} [13], but it is not clear whether these tRNA species contain a Se-nucleotide. It has been suggested that Selenium containing tRNA species in murine leukemia cells are tRNA^{Lys} and that the Se-nucleotide was mSe^{2}t^{6}Ap [20]. It is possible that this mSe^{2}t^{6}Ap is the minor spot of ^{75}Se-nucleotide shown by the arrowhead in Figure 4. Meanwhile, we did not find any evidence that the Se-acceptor nucleoside in the tRNA is mnm^{5}s^{2}U (the incorporation of Se instead of sulfur). Other problems remaining are as follows: the purification of Se-tRNA and its synthase, chemical identification of mnm^{5}Se^{2}U, the position of mnm^{5}Se^{2}U on the tRNA, the recognition sites on the tRNA for the synthase and characterization of the last Se-rich BD fraction.

Acknowledgement

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