Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3010–3013, May 1984
Biochemistry

Occurrence of selenium-containing tRNAs in mouse leukemia cells

(K1210 cells/selenonucleoside/alkaline sensitivity/nuclease sensitivity)

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Communicated by Thressa C. Stadtman, January 27, 1984

ABSTRACT **Selenium incorporation into the polynucleotide structures of tRNAs has been documented in several microorganisms. In the present study, selenium-containing species were isolated from bulk tRNA preparations from ^{75}Se-labeled mouse leukemia cells. The major ^{75}Se-labeled species was similar in size and exhibited the same sensitivity to ribonuclease as did *Escherichia coli* tRNAs. The chromatographic properties of the intact major selenium-containing tRNA species indicated it to be very hydrophobic in character. The selenium component that is unstable at neutral-to-alkaline pH but is relatively stable at acid pH is not an esterified selenoamino acid. HPLC analysis of enzymic digests of the major selenium-containing species detected selenium-containing hydrophobic products (probably selenonucleosides). These properties strongly suggest that the selenium in the mouse leukemia-cell tRNAs is present in the form of a selenium-modified nucleoside.**

The essential role of selenium in animal nutrition is well established. In humans it has been demonstrated that an endemic cardiomyopathy (Keshan disease) prevalent in certain areas of China (1) is correlated with selenium deficiency. Patients on selenium-free total parenteral nutrition regimes in the United States responded favorably to selenium supplementation (2). Selenium also has been claimed to prevent certain types of cancer, to enhance immune responses, and to increase fertility in domestic animals. The discovery of selenium as an essential component of several enzymes provided some molecular basis for its biological function.* In mammals glutathione peroxidase is the only selenium-dependent enzyme so far identified, but it seems unlikely that the complicated biological effects of selenium in man and animals are attributable solely to variations in the level of this enzyme. In fact, at least two other selenoproteins of unknown catalytic activity have been detected in animals. Recently, specific incorporation of selenium into tRNA molecules has been observed in several bacterial species (3–5). Seleno-tRNA^{Glu} and seleno-tRNA^{Lys} are among the most prominent seleno-tRNAs in *Clostridium sticklandii* and *Escherichia coli* (5, 6). Since in a number of instances modified bases in tRNAs have been shown to be involved in regulatory functions of the tRNAs, the selenium-modified bases may have similar roles. Furthermore, some RNA viruses (7) as well as leader mRNAs (8) contain tRNA-like segments that may interact with specific enzymes and, thus, play regulatory roles. Hence, it is reasonable to assume that detailed information concerning selenium-modified tRNAs may contribute to the understanding of the biological roles of selenium. The purpose of the present work was to find out if selenium-containing tRNAs exist in mammalian tissues, and if so, to characterize the selenium components present.

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MATERIALS AND METHODS

Materials. The following were purchased from commercial sources: bulk tRNA and 5S RNA from *E. coli* MRE 600 (Boehringer Mannheim), ribonuclease phy M (P-L Biochemicals), nuclease P1 (Boehringer Mannheim), potato acid phosphatase (Calbiochem–Behring), $H_2{}^{75}SeO_3$ (200 mCi/mg, New England Nuclear; 1 Ci = 37 GBq), RPMI 1630 medium (Flow Laboratories), fetal bovine serum (Hazelton Dutchland, Denver, PA), DEAE-cellulose (Whatman), and Bio-Gel P-100 (Bio-Rad). Plaskon CTFE 2300 powder and Adogen 464 for reversed phase chromatographic system 5 (RPC-5) column packing were gifts from D. Novelli. A preparation of aminoacyl-tRNA synthetases from *E. coli* was a gift from A. Wittwer.

^{75}Se-Labeled tRNAs. The lymphocytic mouse leukemia L1210 cells were grown in culture medium RPMI 1630 with 15% heat-inactivated (30 min at 56°C) fetal bovine serum in the presence of penicillin and streptomycin. At early logarithmic phase, the cultures were supplemented with $H_2{}^{75}SeO_3$ and incubated overnight at 35°C. The total sulfur content of the culture medium was about 1 mM, and the added selenite was 50–100 nM. A small amount of selenium also was supplied in the fetal bovine serum. Two batches of cells were prepared. The initial cell density, final cell density, and amount of $H_2{}^{75}SeO_3$ added to the two batches, respectively, were as follows: batch 1, 3.2×10^5 cells per ml, 7.1×10^5 cells per ml, and 50 nM $H_2{}^{75}SeO_3$ (0.2 mCi/100 ml); batch 2, 5×10^5 cells per ml, 1.2×10^6 cells per ml, and 100 nM $H_2{}^{75}SeO_3$ (0.4 mCi/140 ml). The labeled cells were sedimented by centrifugation, rinsed with Dulbecco's phosphate buffer without Mg^{2+} and Ca^{2+}, and resuspended in buffer A (100 mM sodium acetate, pH 4.5/150 mM NaCl/10 mM $MgCl_2$/1 mM EDTA/2 mM dithiothreitol). Bulk tRNAs were isolated from the ^{75}Se-labeled cells by the method of Holley (9) except that buffer A was used throughout the procedures. The bulk tRNA preparation was subjected to RPC-5 chromatography by the method of Kelmers and Heatherly (10).

Enzymic Hydrolysis of ^{75}Se-Containing tRNA for Nucleoside Analysis. A sample of ^{75}Se-labeled material (5,000 cpm) that was eluted from the RPC-5 column in peak II (see Fig. 1) was mixed with *E. coli* bulk tRNA (0.35 A_{260} unit), evaporated to dryness, and then taken up in 10 μl of 0.1 M ammonium acetate (pH 4.5). The sample was first treated with 2 μl of nuclease P1 (300 units/ml) for 30 min at 37°C and then with 2.5 μl of potato acid phosphatase (0.5 unit/μl) for another 30 min. The whole reaction mixture was then applied to a C_{18} μBondapak column and subjected to HPLC analysis.

RESULTS AND DISCUSSION

In preliminary survey experiments, cultured cell lines (mouse leukemia cells and human leukemia cells) and a pro-

*Stadtman, T. C., Ching, W.-M., Hartmanis, M., Sliwkowski, M., Tsai, L., Wittwer, A. & Yamazaki, S., Fourth International Conference on the Organic Chemistry of Selenium and Tellurium, July 25–29, 1983, Birmingham, England.

tozoan, acanthamoeba, which had been grown in medium supplemented with $H_2{}^{75}SeO_3$, were examined for the presence of selenium-containing tRNAs. Also, mice (Ehrlich ascites tumor-bearing strain BALB/c) that had been injected with $Na_2{}^{75}SeO_3$ were sacrificed after 2 days, and bulk tRNAs from the tumors, liver, kidney, and spleen were isolated and assayed. Various amounts of radioactive selenium were detected in the bulk tRNA preparations from all of these samples. Since the mouse leukemia cell line is easy to culture and the pattern of ^{75}Se profiles on the RPC-5 column is reproducible from batch to batch, this cell line (L1210) was chosen for further study. The selenium content of the bulk tRNA population from L1210 cells cultured in the synthetic medium RPMI 1630 was estimated to be about 1 pmol/A_{260} unit. This amount is significantly lower than those observed (3–5) for several bacterial species (80–300 pmol/A_{260} unit). Likewise, as calculated from the results of Eliceir (11), the sulfur content of tRNA in mouse lymphoma cells is also much less (130 pmol/A_{260} unit) than that of *E. coli* tRNA, in which almost half of the tRNA population are modified by sulfur (830 pmol/A_{260} unit).

An RPC-5 reversed-phase chromatographic profile of ^{75}Se-labeled tRNAs from L1210 cells is shown in Fig. 1. Note that no selenium-containing species was eluted until the end of the gradient (0.85 M NaCl), and only one major ^{75}Se-containing species was detected. This pattern is very different from those observed for certain bacterial species, e.g., *E. coli*, *C. sticklandii*, and *Methanococcus vannielii*. In each of these bacterial species, two or three prominent seleno-tRNAs were eluted from the RPC-5 column with 0.5–0.6 M NaCl, followed by several minor species eluted with higher salt concentrations. Among the major seleno-tRNAs in the bacterial population are glutamate- and lysine-isoaccepting species. Preliminary results indicated that each of the three ^{75}Se peaks (peak I, II, and III of Fig. 1) exhibited a low but detectable amino acid-accepting activity when a mixture of 15 tritiated amino acids was used.

To see if the ^{75}Se-labeled species from the L1210 cells present in the major ^{75}Se-labeled peak (peak II, fractions 112–117, in Fig. 1) was actually a seleno-tRNA species, it was recovered from the column eluent and subjected to the following studies: (*i*) stability as a function of pH and temperature, (*ii*) size measurement, (*iii*) sensitivity to nucleases, and (*iv*) nucleoside analysis.

In the initial experiments, it was noticed that the selenium component in the bulk tRNA preparations from the L1210 cells was very unstable. Accordingly, the stability of the selenium component(s) under various conditions, as measured by ethanol-precipitable ^{75}Se, was investigated. Since the incorporated radioactivity was very alkali-labile, the ^{75}Se-labeled material in the peak II fractions (Fig. 1) that became soluble in ethanol and separated from the tRNA preparation was recovered and chromatographed on an amino acid analyzer column to determine if ^{75}Se-containing amino acids were present. Most of the ^{75}Se applied to the amino acid analyzer column was eluted in fractions near the breakthrough volume and in the final alkali wash, indicating its presence in various breakdown products. Because selenocysteine is unstable and, if initially present, might have been decomposed during subsequent isolation steps, additional samples of the ^{75}Se-labeled material were mixed with carrier selenocysteine and carboxymethylated prior to amino acid analysis. After incubation in 0.35 M Tris·HCl (pH 8.5) at 37°C for 30 min to deacylate esterified amino acids, the radioactive mixtures were treated with KBH_4, carboxymethylated with iodoacetate, and fractionated on an amino acid analyzer column. The carboxymethylselenocysteine peak, which accounted for 64% of the added carrier selenocysteine, contained no ^{75}Se. Failure to detect [^{75}Se]selenocystine, carboxymethyl[^{75}Se]selenocysteine, or [^{75}Se]selenomethionine at the expected elution positions of these amino acids indicated that the radioactivity in peak II fractions was not present in the form of esterified selenoamino acids.

Since the UV absorbance associated with ^{75}Se radioactivity in the peak II fraction of Fig. 1 was low (<0.04 A_{260} unit/ml), *E. coli* bulk tRNA was added as a carrier, and the resulting mixture was subjected to size characterization by gel-filtration chromatography. The elution position of ^{75}Se

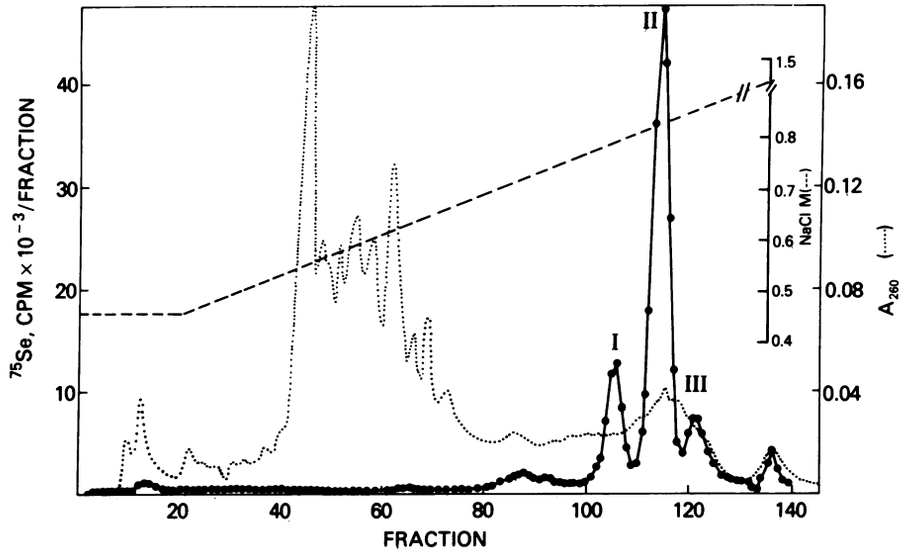

FIG. 1. Reversed-phase chromatography of ^{75}Se-labeled tRNAs from L1210 cells. Bulk tRNA (6.8 A_{260} units; 4 × 10^5 cpm) in 5 ml of buffer B (10 mM sodium acetate, pH 4.5/10 mM magnesium acetate/1 mM EDTA/1 mM dithiothreitol/0.45 M NaCl) was applied to a RPC-5 column (0.9 × 41 cm bed). After the column was washed with 10 ml of buffer B, the absorbed tRNA was eluted with a linear gradient (total volume, 240 ml) of buffer B and buffer C (same as buffer B but containing 0.85 M NaCl). Fractions (2 ml) were collected at a flow rate of 1.0 ml/min. At the end of the gradient, buffer D (same as buffer B but containing 1.5 M NaCl) was pumped through the column to elute strongly absorbed materials. The column pressure slightly increased from 350 pounds per square inch (psi; 1 psi = 6.89 kPa) to 500 psi as the salt concentration increased during the elution. The ^{75}Se content of each fraction was determined in a γ scintillation counter (Beckman 5500). The total recovery of the applied radioactivity in the ^{75}Se-containing peaks labeled I–III (fractions 100–125) was 70%. The last ^{75}Se peak (fractions 134–138) required 1.5 M NaCl for its elution.

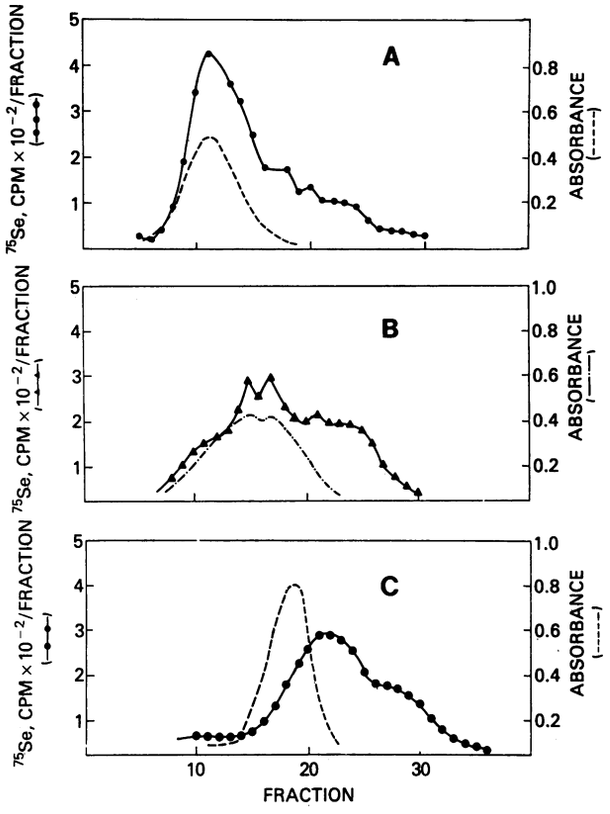

FIG. 2. (*A*) Polyacrylamide P-100 gel filtration profiles of a selenium-containing tRNA species (peak II in Fig. 1) mixed with carrier *E. coli* bulk tRNA: 150 μl of sample (4000 cpm of ^{75}Se; 3.6 A_{260} units, of which 99.5% was the carrier tRNA) was applied to a Bio-Gel P-100 column (effective range, M_r 5000–100,000; 1 × 18 cm column bed) equilibrated with 0.2 M potassium acetate, pH 5.0/1 mM dithiothreitol/1 mM EDTA. Blue dextran was eluted in the fractions between 5 and 7 ml. The recovery of ^{75}Se and absorbance units were 64% and 67%, respectively. (*B*) Polyacrylamide P-100 profile of selenium-containing tRNA species (peak II of Fig. 1) after partial digestion. The partial digestion reaction mixture (125 μl) contained 4000 cpm of ^{75}Se from peak II, 1.75 A_{260} units of *E. coli* bulk tRNA, 5 μmol of sodium citrate (pH 3.5), 1 mM EDTA, and 50 units of ribonuclease phy M. (*C*) Polyacrylamide P-100 profile of selenium-containing tRNA species (peak II of Fig. 1) after complete digestion. The complete digestion reaction mixture (200 μl) contained 4000 cpm of ^{75}Se from peak II, 3.6 A_{260} units of *E. coli* bulk tRNA, 8 μmol of ammonium acetate (pH 4.5), and 20 μg (6 units) of nuclease P1. All incubations were 1 hr at 37°C.

and its correlation with A_{260} absorbancy of the carrier tRNA (Fig. 2*A*) indicate that the selenium-containing material from the L1210 cells has a molecular weight of 25,000, similar to those of *E. coli* tRNAs. To see if the incorporated ^{75}Se was actually present in the polynucleotide structure of a tRNA, the effects of digestion with nucleases were studied. Because of marked instability of the selenium-containing component at alkaline or neutral pH (Table 1), ribonuclease phy M and nuclease P1, which are active at acidic pH, were chosen for either partial digestion (phy M) or complete digestion (P1). The same sample (^{75}Se-labeled peak II of Fig. 1 with carrier *E. coli* bulk tRNA) that had been used for size characterization (Fig. 2*A*) was incubated with ribonuclease phy M, which cleaves RNA almost exclusively at UψN and AψN bonds. The resulting partial digest was subjected to chromatography on the same polyacrylamide P-100 gel filtration column (Fig. 2*B*). The correlation of ^{75}Se with A_{260} absorbancy in the two major peaks eluted in fractions 10 to 20 strongly suggests that the selenium-containing species exhibited the same sensitivity towards ribonuclease as did the *E. coli*

Table 1. Stability of selenium in seleno-tRNAs isolated from L1210 cells

Buffer system	Treatment	% of ethanol-precipitable Se*
H_2O	Boiled 2 min	17†
0.2 M sodium citrate (pH 2.4)	Boiled 2 min	62†
	37°C 1 hr	83†
5 mM sodium acetate, 5 mM magnesium acetate, and 0.25 M sodium chloride (pH 4.8)	Boiled 2 min	26‡
0.2 M sodium acetate (pH 5.0)	37°C 1 hr	72†
0.2 M sodium phosphate (pH 6.9)	37°C 1 hr	14†
0.1 M Tris (pH 8.0)	37°C 1 hr	9.4‡
1.0 M Tris (pH 8.0)	37°C 1 hr	4.4‡

*After each treatment, NaCl was added to give the final concentration of 0.5 M, followed by 3 vol of ethanol; then the sample was stored at −20°C for 2 hr. The amount of ^{75}Se was determined in both supernatant and precipitate.
†Bulk tRNA.
‡Peak II off RPC-5 column (Fig. 1).

tRNAs. The chromatographic profile of the sample after complete digestion with nuclease P1 is shown in Fig. 2*C*. These results demonstrate that the incorporated ^{75}Se is located in the nuclease-sensitive polynucleotide structure. From Fig. 2*C* it is clear that the selenium-containing components resulting from nuclease digestion bind to the column matrix and were eluted later than the normal nucleotides. Nonspecific interaction of selenium compounds with various column matrix has been repetitively observed in this laboratory. This may explain the tailing of ^{75}Se in Fig. 2 *A* and *B*.

To further characterize the selenium-containing components, the sample was digested to nucleosides with nuclease P1 and acid phosphatase at pH 4.5 to avoid decomposition of the alkali-sensitive ^{75}Se-labeled components. The digestion mixture was analyzed by HPLC on a reversed-phase column

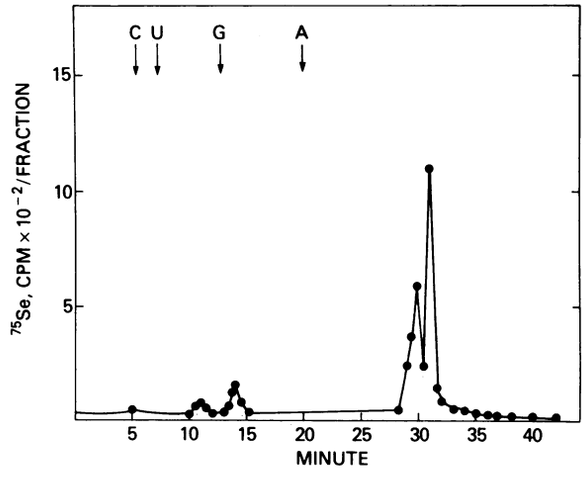

FIG. 3. Reversed-phase HPLC analysis of ^{75}Se-containing species (peak II of Fig. 1) hydrolyzed with nuclease P1 and phosphatase: ^{75}Se sample (5,400 cpm) was mixed with *E. coli* bulk tRNA (0.35 A_{260} unit), digested to nucleosides by nuclease P1 and acid phosphatase, and chromatographed on a Waters μBondapak C_{18} column at room temperature and at the flow rate of 1 ml/min. The column was equilibrated with 10 mM ammonium acetate (pH 5.3) in 3% methanol and eluted with increasing methanol gradients as follows: from 0 to 20 min, 3–20%; from 20 to 25 min, 20–100%; and from 25 to 35 min, 100%. The ^{75}Se in 0.5-ml fractions was determined by scintillation spectrometry after adding 3 ml of Aquasol. Of the applied ^{75}Se, 50–60% was recovered in the fractions that were eluted at 29–31 min. The major nucleosides, C, U, G, and A, were eluted at 5.3, 6.6, 12.3, and 19.2 min, respectively.

(Fig. 3). About 50–60% of the applied radioactivity was recovered in fractions eluted at 29–31 min with a mobile phase of 100% methanol. This delayed emergence of the radioactive material from the column suggests a strong hydrophobic interaction between the selenium component and the column packing material. Certain highly modified thioadenosine derivatives have been shown to exhibit such strong interactions with the C_{18} reversed-phase column (12). Naturally occurring compounds of this type are 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A) and 2-methylthioribosylzeatin (ms^2io^6A), which have been found in the tRNAs of several microorganisms (13, 14) though not in animal cells (15) and *N*-[(9-β-D-ribofuranosyl-2-methylthiopurine-6-yl) carbamoyl]threonine (ms^2t^6A), which is present in $tRNA^{Lys}$ of rabbit liver (16) and *Bacillus subtilis* (17). Selenium analogs of these thioadenosines might be expected to exhibit chromatographic properties similar to that of the selenonucleoside in peak II (Fig. 1). A likely candidate for the major seleno-tRNA in this peak might be a lysine-accepting tRNA with *N*-[9-β-D-ribofuranosyl-2-methylselenopurine-6-yl)carbamoyl]threonine (mSe^2t^6A).

I acknowledge the advice and encouragement offered by Dr. Thressa C. Stadtman during the course of this work. I also am grateful to Dr. Kurt W. Kohn and Ms. Susan Hurstcalderone for providing L1210 cells; to Dr. Cindy N. Oliver for providing human leukemia HL60 cells, and to Dr. Todd M. Martensen for helping with BALB/c mice.

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