Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6345–6348, August 1990
Biochemistry

Biosynthesis of selenium-modified tRNAs in *Methanococcus vannielii*

(seleno-tRNAs/aminoacylation)

MICHAEL POLITINO, LIN TSAI, ZSUZSA VERES, AND THRESSA C. STADTMAN

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by Thressa C. Stadtman, June 7, 1990

ABSTRACT **Selenium-containing nucleosides are natural components of several tRNA species in *Methanococcus vannielii*. In the present study, the incorporation of selenium from $^{75}SeO_3^{2-}$ into these macromolecules was investigated in sonic extracts of *M. vannielii*. Nucleoside analysis of the ^{75}Se-labeled tRNAs from these *in vitro* reaction mixtures demonstrated that the selenium was present in ^{75}Se-labeled nucleosides identical to the two naturally occurring 2-selenouridines produced *in vivo*. Incorporation of selenium into these nucleosides was ATP-dependent and was maximal after 20 min. Addition of *O*-acetylserine enhanced the activity 2- to 3-fold, implicating a role for selenocysteine in the reaction. Added L-selenocysteine could function as a selenium donor, but the D isomer and DL-selenomethionine were inactive. RPC-5 chromatography of bulk tRNA isolated from *M. vannielii* grown on $^{75}SeO_3^{2-}$ separated five major species of seleno-tRNAs. The amino acid-accepting activity of these tRNAs was investigated.**

Selenium-modified nucleosides occur in tRNAs from several bacterial (1–4), mammalian (5), and plant (6) species. The most prominent bacterial selenonucleoside is 5-methylaminomethyl-2-selenouridine; other selenonucleosides have yet to be identified (1, 7). Although the precise biochemical role for this specific modification has yet to be defined, the 2-selenouridine that occurs in the "wobble position" of the anticodons of lysine (8) and glutamate (9) tRNAs may regulate codon–anticodon interactions (8, 9). Little is known about the mechanism of incorporation of selenium into selenonucleosides. A likely precursor of the 2-selenouridine is its sulfur analog (10). Because *Methanococcus vannielii* is a particularly rich source of seleno-tRNAs (1) and also contains considerable amounts of at least two selenoenzymes (11, 12), this bacterial species should possess elevated levels of the enzymes and cofactors required for selenium incorporation processes. The present study describes an *in vitro* system of incorporation of selenium into tRNA and provides a partial characterization of the biosynthesis of selenonucleosides in *M. vannielii*.

MATERIALS AND METHODS

Materials. Radioactive amino acids were purchased from Amersham, and $H_2{}^{75}SeO_3$ was a gift from R. E. Schenter (Westinghouse–Hanford, Richland, WA). Nuclease P1 was obtained from Boehringer Mannheim; Supelcosil C_{18} column was from Supelco; DEAE-cellulose was from Whatman; ATP, adenosine 5′-[α,β-methylene]triphosphate, adenosine 5′-[β,γ-methylene]triphosphate, *O*-acetyl-L-serine, *O*-phospho-L-serine, *O*-succinyl-L-homoserine, acid phosphatase, and DL-selenomethionine were from Sigma. Plaskon CTFE 2300 powder and Adogen 464 for RPC-5 column packing were gifts from G. D. Novelli (Oak Ridge National Laboratory, Oak Ridge, TN). D- and L-selenocysteine were synthesized as described (13).

Growth of *M. vannielii*. *M. vannielii* cells were cultured in a formate mineral salts medium (11) supplemented with 1.0 μM $NiCl_2$, 2 mM cysteine, 1 mM Na_2S, 1.0 μM Na_2SeO_3, or 0.5 μM $H_2{}^{75}SeO_3$ (2 mCi liter; 1 Ci = 37 GBq). Cells were harvested while actively fermenting. Sonic extracts of *M. vannielii* were prepared from cells suspended in 2 volumes (wt/vol) of 50 mM Tris·HCl/10 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol, pH 8.0.

Assay for Incorporation of ^{75}Se into tRNA. The reaction mixture (500 μl) contained 400 μl of *M. vannielii* sonic extract, 45 μCi of SeO_3^{2-}, 5 mM $MgCl_2$, 1 mM dithiothreitol, and other supplements as indicated. Samples were incubated under argon for 20 min at 37°C, and reactions were terminated by adding 500 μl of phenol saturated with buffer A (20 mM sodium acetate/10 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol, pH 4.5). The tRNA was isolated, and incorporated ^{75}Se was determined by measuring the radioactivity in a Beckman γ 5500 counter.

Isolation of tRNA. Cell extracts were gently shaken with equivolume amounts of phenol saturated with buffer A for 1.5 hr. The aqueous layer was removed and re-extracted with phenol. After the second phenol extraction the tRNA in the aqueous layer was precipitated by adding 2 vol of cold ethanol. After several hours at −20°C, the precipitate was collected and resuspended in buffer A, and the procedure was repeated. The second tRNA precipitate in buffer A was applied to a DEAE column equilibrated with buffer A. After being washed with buffer A/0.3 M NaCl, the adsorbed tRNA was eluted with buffer A/1 M NaCl and precipitated with 2 vol of cold ethanol. RPC-5 chromatography of bulk tRNA was done as described (14).

Aminoacylation of tRNA. The reaction mixture (40 μl) contained 100 mM Tricine·KOH (pH 7.5), 5 mM ATP, 20 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM KCl, 228 μg of *M. vannielii* aminoacyl-tRNA synthetase, ^{14}C-labeled amino acids, and 0.1–1.0 A_{260} unit of tRNA. The acceptor activity was assayed by a modification of the procedure of Kelmers *et al.* (15). The reaction was spotted on 2.3-cm Whatman 3-MM filter disks and washed once in cold 10% trichloroacetic acid, twice in cold 5% trichloroacetic acid, and once in ethanol for 15 min. The disks were dried, and trichloroacetic acid precipitable radioactivity was determined by scintillation counting.

Nucleoside Analysis. The ^{75}Se-labeled tRNA was denatured by boiling for 2 min and then treated with nuclease P1 (10 μg) and potato acid phosphatase (50 μg) for 90 min. The reaction mixture was then applied to a Supelcosil C_{18} column and subjected to HPLC analysis using a Spectra-Physics SP8700 solvent-delivery system with a Hewlett–Packard 1040A spectrophotometer detector. Details are given in the legend of Fig. 2.

RESULTS AND DISCUSSION

Because *M. vannielii* must be grown in media supplemented with selenium in order to synthesize essential selenium-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

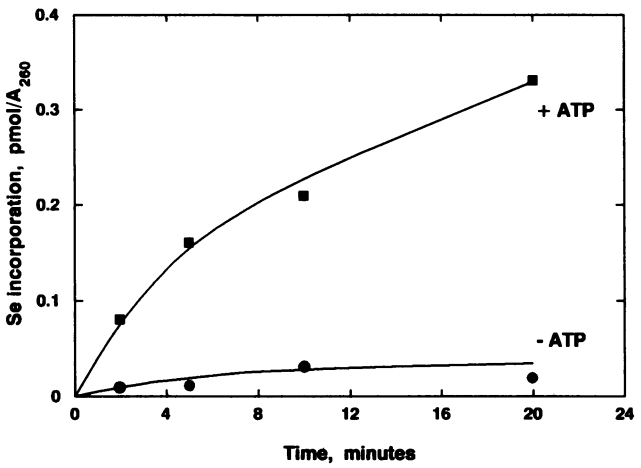

FIG. 1. Time course of incorporation of ^{75}Se into tRNA. Sonic extracts of *M. vannielii* (0.4 ml) were added to the standard assay mixture and incubated at 37°C under argon with 45 μCi of $^{75}SeO_3^{2-}$ and 50 μM SeO_3^{2-} in the presence (■) and absence (●) of 5 mM ATP. At the indicated times the reactions were stopped, the tRNA was isolated, and incorporated radioactivity was determined by γ counting.

dependent enzymes, many of the endogenous tRNAs are already present as the seleno forms, thus greatly reducing the availability of substrate for the *in vitro* incorporation of selenium. A time course of incorporation of selenium into tRNA (Fig. 1) reveals that in the presence of ATP a maximum is attained after 20 min. Although the amount of overall incorporation of ^{75}Se (to 0.5 pmol per A_{260}) is much lower than the level found in bulk tRNA labeled *in vivo* (320 pmol per A_{260}), it is sufficient to explore requirements for the reaction. Upon addition of 10 units of bulk *M. vannielii* tRNA to the reaction mixture, there is an increase of ≈2 pmol in the total amount of selenium incorporated with no change in specific activity (≈1000 cpm per A_{260} unit, 0.25 pmol per A_{260} unit). In the absence of ATP, selenium incorporation is greatly decreased and reaches a maximum at a much lower level during the same 20-min period (Fig. 1). This ATP-dependence of selenium incorporation has been observed with permeabilized *Escherichia coli* cells (10) and more recently with French press extracts of *Salmonella typhimurium* (16).

Increasing the concentration of unlabeled Na_2SeO_3 in the reaction mixture, up to 100 μM, resulted in an increase in the incorporation of selenium into the tRNA (Table 1). Addition of *O*-acetyl-L-serine, which can be converted to selenocysteine in the presence of HSe^- by the action of *O*-acetylserine sulfhydrylase (17), also enhanced the reaction 2- to 3-fold (Table 2). These results implicate a role for selenocysteine as selenium donor in the reaction. Addition of *O*-phosphoserine

Table 1. Effect of increasing Na_2SeO_3 concentration on the incorporation of selenium into tRNA

Added Na_2SeO_3, μM	Se in tRNA	
	cpm/A_{260} unit	pmol/A_{260} unit
0	1354	≈0.0027*
10	1162	0.06
20	902	0.09
50	934	0.23
100	834	0.42

Reaction mixtures (0.5 ml) containing 45 μCi of $^{75}SeO_3^{2-}$, 5 mM ATP, unlabeled selenite as indicated, and the standard assay reagents described in the text were incubated for 20 min at 37°C.

*When only the radioactive tracer (45 μCi) was present, the total amount of selenium added was ≈0.18 nmol. This amount neglects the amount of unlabeled selenium present in endogenous selenium compounds in the enzyme preparation.

Table 2. Stimulation of selenium incorporation into tRNA by *O*-acetylserine

Addition	Se in tRNA	
	cpm/A_{260} unit	pmol/A_{260} unit
None	986	0.25
O-acetyl-L-serine	2599	0.65
O-phospho-L-serine	966	0.25
O-succinyl-L-homoserine	1063	0.27

Reaction mixtures (0.5 ml) contained 50 μM SeO_3^{2-}, 5 mM ATP, and serine or homoserine ester (500 μM) as indicated in addition to the standard assay reagents.

or *O*-succinylhomoserine resulted in no enhancement of activity (Table 2). Because one role of ATP in the reaction is the generation of acetyl-CoA required for formation of *O*-acetylserine, a further requirement for ATP in the presence of added *O*-acetylserine was investigated. As seen in Table 3, there is only a small increase in selenium incorporation when ATP is added in the presence of *O*-acetylserine. However, when ATP is replaced with the ATP analogs, selenium incorporation is significantly reduced (Table 3). These results suggest that there is an ATP-dependence in addition to that required for *O*-acetylserine synthesis, but this requirement is not detectable until the effective concentration of the endogenous ATP is reduced upon addition of the competing ATP analogs. When equimolar concentrations of ATP and analog are present, there is no reduction of selenium incorporation (data not shown).

To further characterize the possible mechanism of selenium incorporation, the nature of the selenium donor was investigated. In the reaction mixtures SeO_3^{2-} is reduced to HSe^- by the added dithiol, and this can serve as donor for the formation of selenocysteine from *O*-acetylserine by enzymes in the crude extract. In view of the enhanced activity seen on *O*-acetylserine addition, which suggests involvement of selenocysteine in the process, the ability of unlabeled selenocysteine to dilute the labeled intermediate was investigated. The results (Table 4) show that without any additional source of selenium, incorporation of ^{75}Se from the added $^{75}SeO_3^{2-}$ is unaffected by adding D-selenocysteine or DL-selenomethionine to the reaction mixture. In contrast, L-selenocysteine addition resulted in a marked decrease in the specific activity of the labeled tRNAs. These results suggest dilution of a labeled selenium donor by the added nonradioactive L-selenocysteine.

Nucleoside analysis of the product of the *in vitro* selenation reaction formed in the absence of *O*-acetylserine (Fig. 2) revealed that the radioactive peaks cochromatographed with the two selenium nucleosides isolated from cells labeled *in vivo* with radioactive selenite. The early peak at 11 min containing 72% of the radioactivity corresponds to the selenium-containing nucleoside, 5-methylaminomethyl-2-sele-

Table 3. ATP-dependence of incorporation of selenium into tRNA in the presence of *O*-acetyl-L-serine

Addition	Se in tRNA	
	cpm/A_{260} unit	pmol/A_{260} unit
None	1522	0.38
ATP	1664	0.42
AD*P*[α,β-CH_2]*P*	808	0.20
AD*P*[β,γ-CH_2]*P*	1116	0.28

All samples contained 50 μM SeO_3^{2-}, 500 μM *O*-acetylserine, and 5 mM ATP or the ATP analogs adenosine 5'-[α,β-methylene]-triphosphate (AD*P*[α,β-CH_2]*P*) and adenosine 5'-[β,γ-CH_2]-triphosphate (AD*P*[β,γ-CH_2]*P*) in addition to the standard assay reagents. In the absence of *O*-acetylserine, ^{75}Se incorporation in the presence of 5 mM ATP was 1108 cpm/A_{260} or 0.28 pmol/A_{260} unit.

Table 4. Determination of the ability of the selenoamino acids to act as a substrate in the incorporation of selenium into tRNA

Addition	^{75}Se in tRNA, cpm/A_{260} unit
None	1203
L-selenocysteine	372
D-selenocysteine	1186
DL-selenomethionine	1269

Each reaction mixture (0.5 ml) contained 5 mM ATP, the indicated selenoamino acid (200 μM), and the standard assay reagents.

nouridine (1). The second peak at 30 min corresponds to an as-yet-unidentified selenonucleoside and represents 14% of the radioactivity. Together the two peaks account for 86% of the radioactivity applied to the column. Analysis of a digest of tRNAs labeled in the presence of added *O*-acetylserine showed a similar chromatographic profile. Because 80% of the applied ^{75}Se was recovered in the two nucleoside peaks, the enhanced incorporation of selenium into tRNAs seen in the *O*-acetylserine-supplemented samples is a result of increased incorporation into the nucleoside. Although the isolated labeled tRNAs were not deliberately subjected to a deacylation step, the contribution of esterified ^{75}Se-labeled selenoamino acids to the radioactivity measured appears negligible.

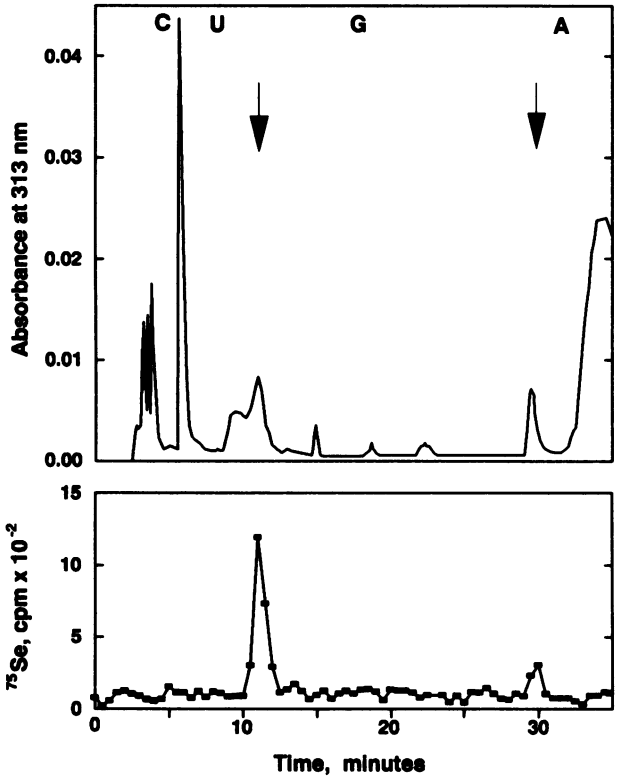

FIG. 2. HPLC nucleoside analysis of *in vitro* ^{75}Se-labeled tRNA from *M. vannielii*. ^{75}Se-labeled tRNA (3.5 A_{260} units; 3500 cpm) was hydrolyzed as described and chromatographed on a Supelcosil C_{18} column at 35°C and flow rate of 1 ml/min. The mobile phase at sample injection was 97% 10 mM ammonium acetate, pH 5.3/3% methanol. The column was eluted with increasing methanol as follows: 10–25 min, 3–5%; 25–30 min, 5–16%; 30–35 min, 16–100% methanol. Fractions (0.5 ml) were collected and monitored at 313 nm, and the ^{75}Se content was determined by γ counting. Elution positions of the four major nucleosides—cytidine, uridine, guanosine, and adenosine—are represented by C, U, G, and A, respectively. Arrows, elution positions of the two naturally occurring selenonucleosides from *M. vannielii*.

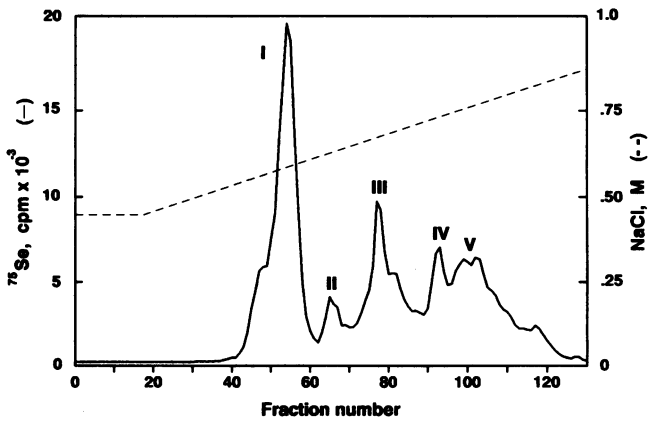

FIG. 3. RPC-5 chromatography of seleno-tRNAs from *M. vannielii*. Bulk tRNA (230 A_{260} units; 500,000 cpm) prepared from *M. vannielii* cells, grown on ^{75}Se-containing media, was suspended in 1.0 ml of buffer B (10 mM sodium acetate/10 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol, pH 4.5) and applied to a RPC-5 column (0.9 × 49 cm). The column was washed with 35 ml of buffer B, and the tRNA was eluted with a linear gradient of 0.45–0.85 M NaCl in buffer B at a flow rate of 1 ml/min. Two-milliliter fractions were collected, and the ^{75}Se content of each fraction was monitored by γ counting. The fractions of peaks I, II, III, IV, and V were pooled, and the aminoacylation of each pool was investigated as described.

RPC-5 chromatography of bulk tRNA isolated from *M. vannielii* cells cultured in medium containing radioactive selenite separated five major selenium-containing tRNA fractions (Fig. 3). The material in each peak was pooled, concentrated, and assayed for amino acid-accepting activity. Previously it had been reported that in *M. vannielii*, selenium content in tRNA correlates linearly with glutamate-accepting activity (1). From the results shown in Table 5 it can be seen that the first two radioactive peaks, I and II, which were eluted with 0.55 and 0.59 M NaCl, respectively, were highly enriched in glutamate-, glutamine-, and lysine-accepting tRNAs. In *E. coli* (4) and *S. typhimurium* (16, 18) these same tRNA species are the major selenouridine-containing species. The three later radioactive peaks, which are unique to *M. vannielii* (1), were also assayed for amino acid-accepting

Table 5. Comparison of the aminoacylation of bulk tRNA with that of the five seleno-tRNA fractions separated by RPC-5 chromatography

Amino acid	Conc., μM	Amino acid incorporated, pmol/A_{260} unit					
		Bulk	Fr I	Fr II	Fr III	Fr IV	Fr V
Ala	73	3.1	6.2	4.4	5.4	14	3.6
Arg	17	1.8	3.8	3.4	9.6	4.4	17
Asn	25	0.5	5.0	4.8	3.2	1.9	3.9
Asp	55	0.5	4.2	1.2	3.8	0	0
Gln	313	1.2	39	47	15	5.2	19
Glu	45	0.6	13	7.4	0	0	3.6
Gly	110	5.1	10	6.4	21	8.7	4.1
His	17	12	2.9	3.3	7.8	3.0	2.3
Ile	37	7.9	11	34	1.8	1.4	1.8
Leu	37	7.0	2.5	2.0	15	6.7	7.1
Lys	17	0.3	6.1	3.6	1.8	2.3	2.3
Phe	24	4.1	2.5	3.7	15	6.7	9.1
Pro	44	0.8	5.3	3.6	1.5	1.7	4.9
Ser	71	1.0	3.6	4.2	3.6	3.2	5.1
Thr	25	0.4	5.5	5.5	2.7	3.1	3.5
Tyr	25	0.4	7.2	0	1.5	1.3	2.4
Val	20	2.2	14	16	6.4	2.5	2.9

Fractions (Fr) eluted from the RPC-5 column were dialyzed against distilled water for 24 hr and concentrated. Amino acid acceptance activity was determined as described. Conc., concentration.

ability and found to be enriched in certain amino acid-accepting tRNAs as compared with the bulk tRNA. However, it has yet to be determined which of these tRNA species actually contains the selenonucleoside.

We are grateful to Mr. Joe N. Davis for technical assistance.

1. Ching, W.-M., Wittwer, A. J., Tsai, L. & Stadtman, T. C. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 57–60.
2. Chen, C.-S. & Stadtman, T. C. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1403–1407.
3. Ching, W.-M. & Stadtman, T. C. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 374–377.
4. Wittwer, A. J. (1983) *J. Biol. Chem.* **258**, 8637–8641.
5. Ching, W.-M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3010–3013.
6. Wen, T.-N., Li, C. & Chen, C.-S. (1988) *Plant Sci.* **57**, 185–193.
7. Wittwer, A. J., Tsai, L., Ching, W.-M. & Stadtman, T. C. (1984) *Biochemistry* **23**, 4650–4655.
8. Wittwer, A. J. & Ching, W.-M. (1989) *BioFactors* **2**, 27–34.
9. Ching, W.-M., Alzner-DeWeerd, B. & Stadtman, T. C. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 347–350.
10. Wittwer, A. J. & Stadtman, T. C. (1986) *Arch. Biochem. Biophys.* **248**, 540–550.
11. Jones, J. B. & Stadtman, T. C. (1981) *J. Biol. Chem.* **256**, 656–663.
12. Yamazaki, S. (1982) *J. Biol. Chem.* **257**, 7926–7929.
13. Tanaka, H. & Soda, K. (1987) *Methods Enzymol.* **143**, 240–243.
14. Kelmers, A. D. & Heatherly, D. E. (1971) *Anal. Biochem.* **44**, 486–495.
15. Kelmers, A. D., Novelli, G. D. & Stuhlberg, M. P. (1965) *J. Biol. Chem.* **240**, 3979–3983.
16. Veres, Z., Tsai, L., Politino, M. & Stadtman, T. C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6341–6344.
17. Dilworth, G. (1982) *J. Labelled Comp. Radiopharm.* **29**, 1197–1201.
18. Kramer, G. F. & Ames, B. N. (1988) *J. Bacteriol.* **170**, 736–743.