ISOLATION AND CHARACTERIZATION OF 2-THIOCYTIDINE FROM A SERINE TRANSFER RIBONUCLEIC ACID OF *ESCHERICHIA COLI*

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1. Introduction

A variety of minor pyrimidine constituents containing sulfur have been discovered in tRNA's from various sources [1–4]. In sequential studies on tRNA's of *E. coli*, we found that $tRNA_3^{Ser}$ contains one mole of 2-thiocytidine. This communication briefly reports the isolation and characterization of 2-thiocytidine from $tRNA_3^{Ser}$ of *E. coli*. This is the first demonstration of the presence of 2-thiocytidine in a specific tRNA.

2. Materials and methods

E. coli $tRNA_3^{Ser}$ was the product used in previous experiments [5,6]. Paper chromatography was carried out by the ascending technique using Toyoroshi No. 51A paper. Thin-layer chromatography and thin-layer electrophoresis were carried out on Avicel SF cellulose (10 × 10 cm). A synthetic sample of 2-thiocytidine was kindly provided by Dr. T.Ueda.

2-Thiocytidine 3′-phosphate was synthesized from 2-thiocytidine [7] as follows. 2-Thiocytidine (100 mg) was phosphorylated with 1.3 equivalents of phosphorus oxychloride in 15 ml of triethylphosphate at 0° for 3 hr with vigorous stirring and then the mixture was kept at 4° for 12 hr. This phosphorylated mixture was then refluxed for 3 hr with 1.5 equivalents of dicyclohexylcarbodiimide in aqueous butan-3-ol in the presence of 4-morpholinodicyclohexylcarboxamidine. The reaction products were fractionated by DEAE-Sephadex A-25 column chromatography (1.1 × 90 cm) with a linear gradient of 0.05 M to 0.70 M triethylammonium bicarbonate buffer (pH 7.5). The fractions containing 2-thiocytidine 2′,3′-cyclic phosphate were collected. 2-Thiocytidine 2′,3′-cyclic phosphate was converted to its 3′-phosphate by digestion with bovine pancreatic ribonuclease (RNAase) I. Final purification was achieved by thin-layer electrophoresis in 0.05 M triethylammonium bicarbonate buffer (pH 7.5) for 15 min at 30 V/cm. The yield of 2-thiocytidine 3′-phosphate from the nucleoside was about 15 percent. The phosphate residue of this synthetic nucleotide can be removed by treatment with *E. coli* alkaline phosphatase, but not with snake venom 5′-nucleotidase.

To test for the presence of 2-thiocytidine 3′-phosphate in $tRNA_3^{Ser}$, 15 A_{260} units of dialyzed $tRNA_3^{Ser}$ were hydrolyzed with RNAase T_2, and the digest was subjected to two dimensional paper chromatography using the system for detection of minor components described by Seno et al. [8].

2-Thiocytidine was isolated from $tRNA_3^{Ser}$ as follows. A $tRNA_3^{Ser}$ preparation (415 A_{260} units) was extensively hydrolyzed with RNAase T_1 and chromatographed on a column of DEAE-Sephadex A-25 with a linear gradient of NaCl in the presence of 7 M urea at pH 7.5. Analysis of the nucleotide in each fraction with RNAase T_2 revealed that 2-thiocytidine 3′-phosphate was present in an oligonucleotide fraction eluted in the last peak. This oligonucleotide fraction was hydrolyzed with bovine pancreatic RNAase I and chromatographed on a column of DEAE-Sephadex A-25 (0.3 × 100 cm) with a linear gradient of 0.14 M to 0.70 M triethylammonium bicarbonate buffer (pH 7.5). Cp, Up, Gp and

ApCp were eluted in order from the column and then the fraction containing 2-thiocytidine 3′-phosphate was eluted with a concentration of 0.28 M triethylammonium bicarbonate. To remove a contaminating substance which showed a spectrum similar to that of Ap, this fraction was hydrolyzed with RNAase T_2 and one third of the hydrolyzate was chromatographed on a thin-layer plate with isobutyric acid-conc. ammonium hydroxide-water (66 : 1 : 33, v/v/v) as solvent. The yield of the thionucleotide was 0.5 A_{260} (at neutrality). The remaining two thirds of the hydrolyzate was treated with *E. coli* alkaline phosphatase and subjected to thin-layer chromatography with butan-3-ol-water (86 : 14, v/v) as solvent, yielding 1.1 A_{260}units (at neutrality) of the thionucleoside.

3. Results

After digestion of $tRNA_3^{Ser}$ with RNAase T_2, a spot of 2-thiocytidine 3′-phosphate was detected at a position between Cp and ψp in a yield nearly equivalent to that of rTp. A spot of 2′,3′-cyclic phosphate of *N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl] threonine was also detected close to this spot [6]. The R_f value of the former was slightly higher in the second run so that the two spots were clearly separated.

The nucleoside and the nucleotide obtained from $tRNA_3^{Ser}$ were directly compared with chemically synthesized 2-thiocytidine and 2-thiocytidine-3′-phosphate, respectively. Fig. 1 shows a comparison of the ultraviolet absorption spectra of the nucleoside obtained from $tRNA_3^{Ser}$ with those of a synthetic sample of 2-thiocytidine. The two spectra are identical in all respects. The identity of the nucleoside was confirmed by thin-layer chromatography in several solvent systems, as shown in table 1. In all solvent systems, the R_f values of the nucleoside preparation were the same as those of the synthetic specimen. Fig. 1 also shows that the ultraviolet absorption spectra of the nucleotide obtained from $tRNA_3^{Ser}$ were identical with those of a synthetic sample of 2-thiocytidine 3′-phosphate. The identity was also confirmed by thin-layer chromatography and thin-layer electrophoresis. In three solvent systems and under two different conditions of electrophoresis, the nucleotide preparation had the same mobilities as the synthetic specimen, as seen in table 2.

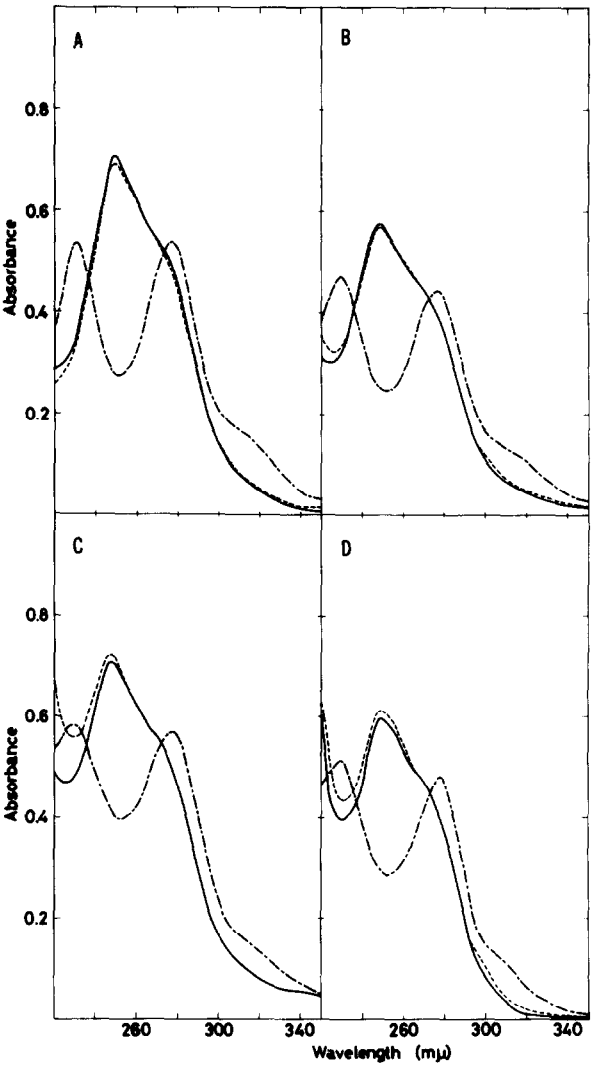

Fig. 1. Ultraviolet absorption spectra of (A) a synthetic sample of 2-thiocytidine, (B) the nucleoside obtained from $tRNA_3^{Ser}$, (C) a synthetic sample of 2-thiocytidine 3′-phosphate and (D) the nucleotide obtained from $tRNA_3^{Ser}$. -.-.-. pH 2, —— pH 7 and - - - - - pH 13.

4. Discussion

The isolation of this sulfur-containing pyrimidine from unfractionated *E. coli* tRNA has been reported by Carbon et al. [2, 4]. Their identification of ^{35}S-labeled nucleotide was, however, based only on its ultraviolet absorption spectra and its conversion to cytosine by acid hydrolysis. Our present results clearly establish the structure of this minor constituent con-

Table 1
Thin-layer chromatographic mobilities of 2-thiocytidine and other nucleosides.

Compound	R_f Value				
	A	B	C	D	E
2-Thiocytidine	0.19	0.55	0.59	0.44	0.37
Nucleoside from $tRNA_3^{Ser}$	0.19	0.54	0.59	0.44	0.37
Cytidine	0.10	0.64	0.50	0.42	0.32
Uridine	0.37	0.49	0.61	0.47	0.55

Solvent system:
A. tert-Butanol-water (86 : 14, v/v)
B. Isobutyric acid-0.5 N ammonium hydroxide (5 : 3, v/v)
C. 1-Propanol-conc. ammonium hydroxide-water (55 : 10 : 35, v/v/v)
D. 1-Butanol-water-acetic acid (4 : 2 : 1, v/v/v)
E. 2-Propanol-conc. hydrochloric acid-water (70 : 15 : 15, v/v/v)

Table 2
Thin-layer chromatographic and electrophoretic mobilities of 2-thiocytidine 3′-phosphate and other nucleotides.

Compound	R_f Value			Migration from origin (cm)	
	E	F	G	H	I
2-Thiocytidine 3′-phosphate	0.53	0.37	0.05	+ 1.9	+ 4.4
Nucleotide from $tRNA_3^{Ser}$	0.54	0.37	0.05	+ 1.9	+ 4.4
Cytidine 3′-phosphate	0.47	0.44	0.11	+ 1.5	+ 4.6
Uridine 3′-phosphate	0.72	0.29	0.09	+ 2.4	+ 4.6

Solvent systems:
E. as in table 1
F. Isobutyric acid-conc. ammonium hydroxide-water (66 : 1 : 33, v/v/v)
G. Ethanol-1 M ammonium acetate buffer at pH 3.7 (7 : 3, v/v)
Electrophoretic conditions:
H. 0.05 M Ammonium formate buffer at pH 3.7 for 15 min at 30 V/cm
I. 0.05 M Triethylammonium bicarbonate buffer at pH 7.5 for 15 min at 30 V/cm.

taining sulfur from *E. coli* $tRNA_3^{Ser}$ as 2-thiocytidine by direct comparison with chemically synthesized 2-thiocytidine. Its structure was confirmed by direct comparison of its 3′-phosphate.

We have purified another species of $tRNA^{Ser}$, $tRNA_1^{Ser}$, whose codon response is different from that of $tRNA_3^{Ser}$ ($tRNA_1^{Ser}$ recognizes codons of the UC series, whereas $tRNA_3^{Ser}$ recognizes those of AGU and AGC) [9]. This $tRNA_1^{Ser}$ does not contain 2-thiocytidine, but contains one mole of 4-thiouridine [10]. It would be interesting to know whether 2-thiocytidine is located at the same position as 4-thiouridine in tRNA, since all of 4-thiouridine residues are located at a common site between the dihydrouracil- and CCA-terminal arms in the sequences of tRNA's reported [11–18]. Sequential studies on $tRNA_3^{Ser}$ to establish the exact location of 2-thiocytidine are in progress.

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