PRESUMED ANTICODON STRUCTURE OF GLUTAMIC ACID tRNA FROM E. coli: A POSSIBLE LOCATION OF A 2-THIOURIDINE DERIVATIVE IN THE FIRST POSITION OF THE ANTICODON.

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Carbon et al. previously reported isolation of 5-methylaminomethyl-2-thiouridine from unfractionated E. coli tRNA as a new minor component (1). However, its exact location in the nucleotide sequence of a particular tRNA is not yet known. In addition, its biological role in tRNA is not understood. This paper reports that a 2-thiouridine derivative, tentatively characterized as 5-methylaminomethyl-2-thiouridine (referred to as N hereafter) was isolated from purified E. coli $tRNA_2^{Glu}$ and sequence analysis of the oligonucleotide containing N revealed that this oligonucleotide was possibly the anticodon loop and this minor nucleoside was located in the first position of the anticodon.

MATERIALS AND METHODS

Preparation of E. coli $tRNA_2^{Glu}$: Unfractionated E. coli B tRNA was first fractionated by DEAE-Sephadex A-50 column chromatography as described previously (2). Glutamic acid tRNA was separated into two fractions by this procedure. The eluate containing the second major peak of $tRNA_2^{Glu}$ was further subjected to benzoylated DEAE-cellulose column chromatography, as described previously (3). $tRNA_2^{Glu}$ was rapidly eluted from the column. Final purification of $tRNA_2^{Glu}$ was achieved by reverse phase partition chromatography at neutral pH (4). This purified $tRNA_2^{Glu}$ could accept 1.04 mμmole of ^{14}C-glutamic acid per 1 O.D. unit of tRNA. Details of this purification procedure will be published elsewhere.

Large scale isolation of Np and N: E. coli $tRNA_2^{Glu}$ (1000 O.D. units) was incubated with 1.3 mg of pancreatic RNase in 6 ml of 0.02 M Tris-HCl (pH 7.5) for 16 hr at 37°C. The hydrolyzate was fractionated by DEAE-Sephadex A-25 column chromatography as described previously (5). The 2-thiouridine derivative (N) was found in the fractions containing NpUp (14 O.D. units) and UpNpUp (20 O.D. units). Six O.D. units of NpUp were desalted, completely hydrolyzed with 0.05 mg of RNase T_2 and applied to a column of Dowex 1 (x 2). Np was eluted as a single homogeneous peak using 0.05 M formic acid as solvent. The yield of Np was 2.2 O.D. units. Nucleoside, N was prepared from Np by treatment with E. coli alkaline phosphatase as described previously (5).

Materials: RNase T_1 and T_2 were purchased from Sankyo Co. Bovine pancreatic RNase (1-A) was obtained from Worthington Biochemical Co. Silkworm nuclease (6) was a gift from Dr. J. Mukai of Kyushu University. Trinucleoside diphosphate, G-A-A and G-A-G were gifts from Dr. T. Ukita of the University of Tokyo. 2-Thiouridine was synthesized as described by Brown et al. (7). 5-Dimethylaminomethyluridine was synthesized by a procedure similar to that described by Kochetokov et al. (8).

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Abbreviation: Np, 3'-nucleotide of N; m^2A, 2-methyladenosine; O.D. units refers to absorbance units measured in a 1 ml cuvette of 1-cm light path at 260 mμ at neutrality

RESULTS

Characterization of the 2-thiouridine derivative in E. coli $tRNA_2^{Glu}$

Approximately 30 O.D. units of E. coli $tRNA_2^{Glu}$ were completely hydrolyzed with excess RNase T_2. The hydrolyzate was assayed by two-dimensional paper chromatography to characterize minor components, as described previously (5). In addition to 1 mole of ribothymidylic acid, 2 moles of pseudouridylic acid and 1 or 2 moles of dihydrouridylic acid, two unusual nucleotides were detected in the hydrolyzate. One of these was identified as 2-methyladenylic acid, and details of its characterization will be published separately (9). The present communication is on the characterization of the other minor nucleotide, Np. Its ultraviolet absorption spectra at different pH values suggested that it was a 2-thiouridine derivative. 5-Methylaminomethyl-2-thiouridine was previously isolated from E. coli unfractionated tRNA by Carbon et al. (1), so studies were made to see whether N was identical with 5-methylaminomethyl-2-thiouridine. As shown in Fig. 1a and b, the ultraviolet absorption spectra of N are identical with those of authentic 2-thiouridine. However, it behaved differently from 2-thiouridine on thin-layer chromatography and electrophoresis. N had a lower Rf value than 2-thiouridine on thin-layer chromatography with solvent B suggesting that it contains an additional positive charge. Confirmation of this extra positive charge was obtained by thin-layer electrophoresis under neutral or acidic conditions. Np and N, respectively behaved like Cp and C rather than like Up and U on thin-layer chromatography and electrophoresis (Table I). Moreover, it was found that Np was eluted just before Cp from a column of Dowex 1. This is a characteristic property of 5-methylaminomethyl-2-thiouridine reported by

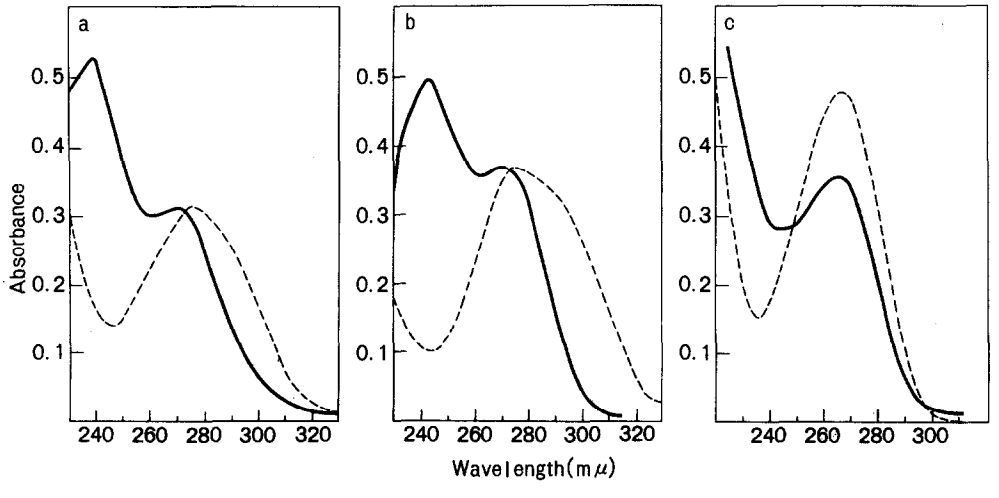

Fig. 1. Ultraviolet absorption spectra of (a) 2-thiouridine, (b) N and (c) desulfurized N. pH 2.0, -----; pH 12.0, ———.

Table I. Relative chromatographic mobilities and electrophoretic mobilities of N, Np and related compounds.

	Thin-layer chromatography Rf in solvent system		Electrophoresis R2',(3')UMP	
	A	B	pH 7.5	pH 2.9
Nucleotide Np	0.33	0.33	0.83	0.0
2',(3')UMP	0.21	0.60	1.0	1.0
2',(3')CMP	0.36	0.39	0.90	0.0
Nucleoside N	0.56	0.18	0.0	
2-Thiouridine	0.51	0.47	0.39	
Uridine	0.43	0.50	0.0	
Cytidine	0.50	0.28		

The solvent systems used were: A, isobutyric acid-0.5 M NH_4OH (5:3, v/v); B, isopropanol-conc. HCl-water (70:15:15, v/v/v). Paper electrophoresis was carried out either in 0.05 M triethylammonium bicarbonate buffer, pH 7.5 or in 0.05 M ammonium formate buffer, pH 2.9.

Carbon et al. (1). To characterize the structure of N further, it was desulfurized by treatment with cyanogen bromide, followed by alkaline hydrolysis, following the procedure used for desulfurization of 4-thiouridine (10). The UV spectra of the

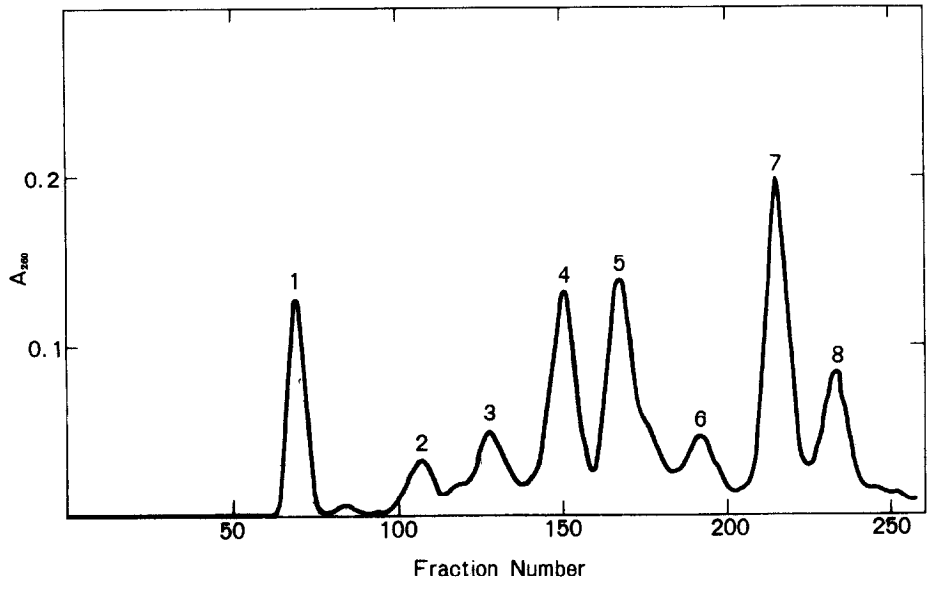

Fig. 2. Separation of oligonucleotides after limited digestion of peak 10-1 with silkworm nuclease. 12 O.D. units of peak 10-1 were incubated with 2.5 μg of silkworm nuclease in 1.5 ml of 0.05 M Na_2CO_3-$NaHCO_3$ buffer (pH 10.5), 0.1 M NaCl and 0.5 mM magnesium acetate at 37°C for 1 hr. The hydrolyzate was applied to a column of DEAE-Sephadex A-25 (0.4 x 40 cm). Elution was achieved with a linear salt gradient obtained by placing 200 ml of Tris-HCl (pH 7.5)-7 M urea in the mixing chamber and 200 ml of Tris-HCl (pH 7.5)-7 M urea-0.4 M NaCl in the reservoir. Fractions of 1 ml of effluent were collected.

product were quite similar to those of authentic 5-dimethylaminomethyluridine ($\lambda_{max}^{0.1\ N\ HCl}$: 267 mμ, ε=9000; $\lambda_{max}^{0.1\ N\ NaOH}$: 266 mμ, ε=6400), indicating that N was a 5-alkylaminomethyl-2-thiouridine (Fig. 1c). Thus, this evidence all strongly suggests that N is 5-methylaminomethyl-2-thiouridine.

Nucleotide sequence of the oligonucleotide containing the 2-thiouridine derivative

E. coli $tRNA_2^{Glu}$ (1000 O.D. units) was extensively hydrolyzed by RNase T_1 and the hydrolyzate was chromatographed on a column of DEAE-Sephadex A-25 with a linear gradient of NaCl in the presence of 7 M urea, as described previously (5). The fractions in each oligonucleotide peak were pooled, desalted and subjected to either column chromatography at pH 2.9 or paper chromatography. Base analysis of each purified oligonucleotide indicated that the 2-thiouridine derivative was only found in peak 10-1 and peak 13. The nucleotide sequence of the oligonucleotide peak 10-1 was determined, but not that of the oligonucleotide peak 13 since the latter was a minor fraction, known to be due to insufficient hydrolysis with RNase. The oligonucleotide peak 10-1 was partially hydrolyzed by silkworm nuclease, and the resulting oligonucleotides were separated by DEAE-Sephadex column chromatography, as shown in Fig. 2. The nucleotide sequence of each oligonucleotide was determined by the conventional technique, using several enzymes such as snake venom phosphodiesterase, *E. coli* alkaline phosphatase, pancreatic RNase and RNase T_2. From the results, together with these on base analysis of peak 10-1, the nucleotide sequence of oligonucleotide peak 10-1 was unambiguously determined as CCCUNUCm^{2}ACG, as summarized in Fig. 3.

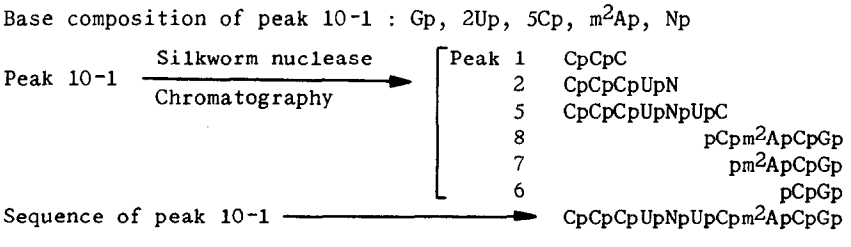

Fig. 3. Scheme for determination of the nucleotide sequence of the oligonucleotide peak 10-1.

Codon recognition properties of $tRNA_2^{Glu}$

Table II shows the binding of ^{14}C-glutamyl $tRNA_2^{Glu}$ to ribosomes in the presence of the trinucleoside diphosphates, G-A-A and G-A-G. When tRNA-ribosome binding experiments were carried out in the presence of 0.02 M Mg^{2+} ion, both G-A-G and G-A-A caused considerably stimulation of the binding of ^{14}C-glutamyl $tRNA_2^{Glu}$ to ribosomes. However, when the Mg^{2+} concentration in the incubation mixture was

Table II. Stimulation of binding of ^{14}C-glutamyl-tRNA$_2^{Glu}$ to ribosomes by G-A-A and G-A-G in the presence of 0.01 or 0.02 M Mg^{2+}.

Template		^{14}C-glutamyl-tRNA bound to ribosomes	
		0.01 M Mg^{2+}	0.02 M Mg^{2+}
None	cpm/tube	42	67
G-A-A	Δcpm/tube	370	1680
G-A-G		68	1010

The reaction mixture contained 2680 cpm of ^{14}C-glutamyl-tRNA$_2^{Glu}$, 1 O.D. unit of ribosomes and 0.1 O.D. unit of G-A-A or G-A-G in a final volume of 0.05 ml. The procedures for preparation of ^{14}C-glutamyl-tRNA and assay of aminoacyl-tRNA binding to ribosomes were described previously (19).

reduced to 0.01 M, the binding of ^{14}C-glutamyl tRNA$_2^{Glu}$ was stimulated by G-A-A, but not by G-A-G.

DISCUSSION

The oligonucleotide peak 10-1 was probably derived from the anticodon loop of tRNA$_2^{Glu}$. The sequence fits very well with the general nature of the anticodon structure, as shown in Fig. 4. If NUC is assumed to be an anticodon, U is next to the 5'-end of the anticodon and m^2A, namely alkylated purine nucleoside is next to the 3'-end of the anticodon. This is the sequence of the anticodon loop common to most tRNA's (See review by Zachau (11), for example). In addition, sequential analysis of other major oligonucleotides derived from an RNase T_1 digest of tRNA$_2^{Glu}$ showed that no other oligonucleotides except peak 10-1 contained the sequence matching the anticodon structure of tRNA^{Glu}.

In tRNA-ribosome binding experiments, E. coli tRNA$_2^{Glu}$ is predominantly recognized by GAA, when the incubation is carried out in 0.01 M Mg^{2+}. It is likely, although not yet proved, that this unique characteristic of single codon recognition

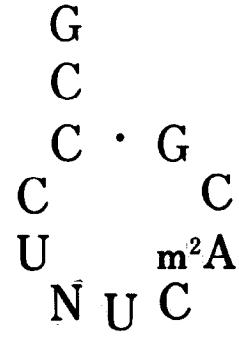

Fig. 4. Structure of the presumed anticodon loop of E. coli tRNA$_2^{Glu}$.

is actually involved in protein synthesis in vivo, since it is known that the binding of tRNA to ribosomes at low Mg^{2+} concentration, rather than at high Mg^{2+} concentration in vitro indicates the true codon recognition in vivo (12). According to the wobble theory proposed by Crick (13), uracil in the first position of the anticodon can form base pairs with both adenine and guanine in the third letter of codons. In the case of the 2-thiouridine derivative in which C-2 oxygen is replaced by sulfur, a stable N-G pair may not be formed, since an S·····H hydrogen bond is weaker than an O·····H hydrogen bond (14). However, N can form a stable base pair with A, since the N-3 hydrogen and C-4 oxygen in N are still available. Modification of the 5'-position of the 2-thiouridine residue might facilitate this tendency.

The specific role of the 2-thiouridine derivative in codon-anticodon base pairing seems to be common in tRNA's from other sources. Yoshida et al. recently showed independently that 2-thiouridine-5-acetic acid methyl ester was also located in the first position of the anticodon of yeast $tRNA_3^{Glu}$ (15). This yeast $tRNA_3^{Glu}$ is recognized only by GAA, not by GAG (16). In this connection, it is interesting to note that a 2-thiouridine derivative was also found in partially purified $tRNA^{Lys}$ from E. coli (M. Saneyoshi and S. Nishimura, unpublished). In addition, recent studies on rat liver $tRNA_3^{Glu}$ and $tRNA_2^{Lys}$ also showed that these tRNA's contained another 2-thiouridine derivative, 5-methyl-2-thiouridine which had not previously been isolated from natural sources (17). It should also be mentioned that rat liver $tRNA_3^{Glu}$ and $tRNA_2^{Lys}$ are predominantly recognized by GAA and AAA, respectively (18).

Another possible explanation for the function of the 2-thiouridine derivative in the first position of tRNA is to prevent miscoding in protein synthesis. In the case of $tRNA^{Leu}$ (for UUA and UUG), $tRNA^{Gln}$, $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA^{Arg}$ (for AGA, and AGG), wobbling of U in the first position of the anticodon with U or C, if present, should cause miscoding which is lethal to cells. 2-Thiouridine derivative instead of uridine in the first position of the anticodon may prevent mispairing with the third letter of U or C. If so it should be possible to isolate the 2-thiouridine derivative from the above mentioned tRNA's as well as $tRNA^{Glu}$ and $tRNA^{Lys}$ This possibility remained to be tested.

Further sequential analysis of E. coli $tRNA_2^{Glu}$ to establish the total primary sequence to determine the exact location of the 2-thiouridine derivative are in progress.

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