E. COLI TYROSINE TRANSFER RNA: CHEMICAL MODIFICATION OF THIOURIDINE TO URIDINE

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One of the common features of the six E. coli transfer RNA's (tRNA's) whose sequences are known is the occurrence of the minor nucleoside 4-Thiouridine (S) (Yaniv and Barrell, 1969 and references therein). The role of S, which is located at a specific position (nucleoside number 8 from the 5'-end) in all these tRNA's and in addition also in number 9 in E. coli $tRNA^{Tyr}$ remains unknown. A direct approach to determine the role of S in the overall function of these tRNA's would be to convert S to its presumed precursor, uridine (Lipsett and Peterkofsky, 1967), and to compare the properties of the modified tRNA with that of the native tRNA. In this communication, we report a general method for the modification of S to U using cyanogen bromide. Application of this reaction on purified E. coli $tRNA^{Tyr}$ and $tRNA^{fMet}$ has shown that the biological properties of these tRNA's are not affected to any appreciable extent.

Several groups (Ziff and Fresco, 1968; Hayatsu and Yano, 1969; Scheit, 1968; Pleiss, et al., 1969; Burton, 1967) have recently described various methods, different from the one described here, for the conversion of S to U or cytidine (C). Application of these methods on a purified species of tRNA for the study of structure - function relationships in tRNA were not described. Saneyoshi and Nishimura (1967 and in press) have modified S to uridine -

4 Thiocyanate in E. coli $tRNA^{Tyr}$ and studied the effect of such modification on the properties of the tRNA.

Materials and Methods

4-Thiouridine was purchased from Sigma Chemical Company. E. coli $tRNA^{Tyr}$ and $tRNA^{fMet}$ were purified in two steps by chromatography on columns of (a) benzoylated DEAE-cellulose and (b) DEAE-Sephadex (Walker and RajBhandary, unpublished). The tRNA's used in the present work were around 90% pure.

Paper chromatography was carried out on Whatman No. 1 paper using the descending technique. The solvent systems used were: A, 1-propanol-concentrated ammonium hydroxide - water (55:10:35, v/v) and B, isobutyric acid - concentrated ammonium hydroxide - water (66:1:33:, v/v; pH 3.7). Thin layer chromatography was carried out on silica gel plates using 1-butanol - water (86:14, v/v) as the solvent. The Rf values obtained in the thin layer system were: 4-thiouridine disulphide, 0.28; uridine, 0.50; uridine 4-thiocyanate, 0.67; and 4-thiouridine, 0.80.

Oligonucleotides were characterized by further degradation as described previously (RajBhandary, et al., 1968).

^{14}C-Tyrosine used had specific activities of either 50 or 362 mc/mmole. Ribosomes and S-100 (supernatant factors) were prepared from E. coli MRE600. The S-100 was freed of tRNA by passing through a column of DEAE-cellulose. In vitro protein synthesis was followed as described previously (Morgan, et al., 1966) using ribopolynucleotides containing specific repeating nucleotide sequences.

Results

(a) Modification of 4-thiouridine-With the ultimate goal of an application of this reaction to tRNA, initial experiments were carried out in phosphate buffer pH 5.0-8.5 and at various temperatures. The optimum conditions thus derived were heating a solution of S with an excess of cyanogen bromide at pH 8.5 at 100°C for 3-4 minutes. Under these conditions the conversion of S to U was quantitative and no side products were detected by thin layer chromatography. The reaction probably proceeded through the intermediate formation of uridine-4-thiocyanate since (i) uridine 4-thiocyanate is produced as the predominant product by reaction of S with cyanogen bromide at pH 6.5 and 37° C for 30 minutes or at pH 8.5 for a few minutes; uridine 4-thiocyanate thus produced had identical Rf, ultraviolet absorption spectrum (λ_{max} 250 mμ and 310 mμ)

and infra red spectrum (peak at 2175 cm^{-1}, characteristic of -S-C≡N) to that obtained from the action of aqueous potassium cyanide on 4-thiouridine disulphide, and (ii) uridine is produced upon heating uridine 4-thiocyanate under similar conditions.

(b) Modification of S in E. coli $tRNA^{Tyr}$ - A solution of $tRNA^{Tyr}$ (150 OD_{260} units and 5 OD_{330} units) in 10 ml. of 0.02 M potassium phosphate pH 8.5 was mixed with cyanogen bromide (50μmoles) and the mixture was heated at 100°C for 4 minutes. The solution was then chilled and dialyzed against water in the cold. Evidence for the quantitative conversion of S to U can be summarized as follows: (i) complete loss of absorption at 334 mμ due to S; (ii) no absorption at 320 mμ indicating the absence of any 4-thiouridine disulphide or uridine -4-thiocyanate; and (iii) as described below, the modified $tRNA^{Tyr}$ upon degradation with T_1-RNase yielded a new hexanucleotide U-U-C-C-C-G- instead of the S-S-C-C-C-G-, present in a similar digest from the native tRNA (RajBhandary, et al., 1969). The same hexanucleotide could be obtained by modification of S-S-C-C-C-G- with cyanogen bromide.

(c) Degradation of modified $tRNA^{Tyr}$ with T_1-RNase- The profiles obtained upon chromatography of a T_1-RNase digest on the native and modified $tRNA^{Tyr}$ are shown in Fig. 1 (A and B respectively). The chromatographic patterns are qualitatively and quantitatively similar except in the following: (i) Peak 12 characterized as the dimer of the hexanucleotide S-S-C-C-C-G- (Harada, Kimura, Nishimura, Chang, Gross, Walker, and RajBhandary, manuscript in preparation) which was present in the native tRNA is mostly absent in the modified tRNA. (ii) Concomittant with the absence of this peak is the appearance of a new hexanucleotide characterized as U-U-C-C-C-G- in peak 8 of Fig. 1B.

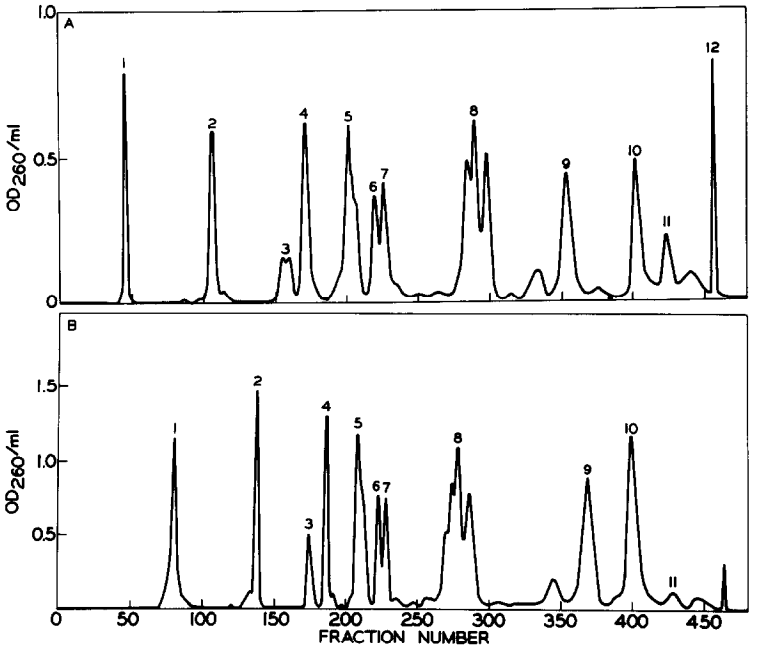

Fig. 1 Chromatography of a T_1-RNase digest of (A) 100 OD_{260} units of native and (B) 150 OD_{260} units of modified E. coli $tRNA^{Tyr}$ on columns (0.7 x 100 cm) of DEAE-cellulose (chloride form). Conditions for digestion with T_1-RNase were as described previously (RajBhandary, et al., 1968) Elution in each case was with a linear gradient (0 → 0.4M) of NaCl (total volume of gradient was 1200 ml) containing 7M urea and 0.02M Tris-HCl (pH 7.3). Fractions approximately 2.5 ml were collected, the pooled peaks were freed of urea and salt by chromatography on columns of Biogel P2 and the oligonucleotides were characterized.

This hexanucleotide was separated from the other three hexanucleotide components of peak 8 by paper chromatography in solvent B. Degradation of this hexanucleotide with pancreatic RNase yielded Up, Cp and Gp in a ratio of 1.9:2.9:1.0. (iii) Peak 11 which is present in Fig. 1A is largely absent from Fig. 1B. A corresponding increase in the relative amount of peak 10 in Fig. 1B suggests that the material in peak 11 represents the hydrogen bonded oligonucleotide complex (RajBhandary, et al., 1969) between material in peak 10 (a nonadecanucleotide from the 3'-end of tRNA) and a heptanucleotide pG-G-U-G-G-G-G- from the 5'-end of the tRNA, the amount of such a complex obtained

in a digest being known to be variable. (iv) A shift in the position of peak 9 in Fig. 1B relative to that of Fig. 1A is observed. Preliminary experiments suggest that the minor nucleoside G^* which is present in this peak (Goodman, et al., 1967) was also modified resulting in the loss of a positive charge known to be present in G^* (Harada, et al., manuscript in preparation).

(d) Aminoacid acceptor activity of modified E. coli $tRNA^{Tyr}$ -

The modification reaction described above causes little loss in the tyrosine acceptor activity of the tRNA. The results described in Table I show that tRNA in which at least 90% of S has been converted to U retains at least 85% of aminoacid acceptor activity.

(e) Use of modified $tRNA^{Tyr}$ in in vitro protein synthesis -

The ability of modified E. coli $tRNA^{Tyr}$ to participate in protein synthesis was followed by using poly r(U-A-C-) as messenger RNA. The assay specifically measured the amount of input ^{14}C-Tyr-$tRNA^{Tyr}$ which could participate in the overall transfer reaction to form polytyrosine. The results (Fig. 2)

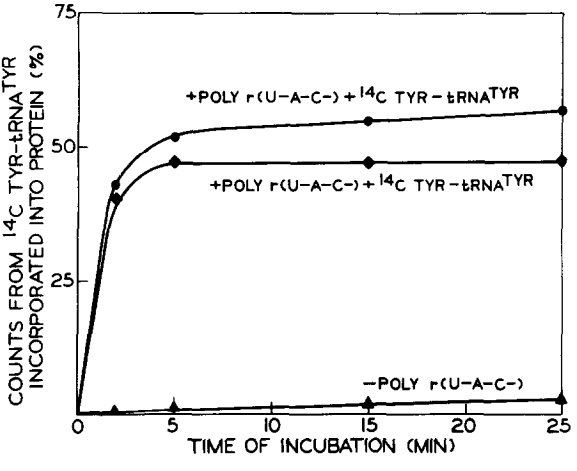

Fig. 2 Incorporation of ^{14}C-Tyr from ^{14}C-Tyr-$tRNA^{Tyr}$ into polytyrosine as directed by poly r(U-A-C-). Poly r(U-A-C-) was synthesized using poly d(T-A-C-): poly d(G-T-A-) as template for RNA polymerase. The assay mixture (0.125 ml) contained poly r (U-A-C-) (12 mμ moles), ribosomes (3.1 OD_{260} units), S-100 (33 μg) and ●, native E. coli ^{14}C-Tyr-$tRNA^{Tyr}$ (0.117 mμ moles; 46,800 cpm) or ◆, modified E. coli ^{14}C-Tyr-$tRNA^{Tyr}$ (0.111 mμ moles; 44,000 cpm). Aliquots (25 μl) were removed at various times and the amount of alkali stable and acid precipitable radioactivity was measured.

indicate that the modified $tRNA^{Tyr}$ is fully functional for protein synthesis; the amount of input modified $tRNA^{Tyr}$ utilized (48%) being comparable to that of native $tRNA^{Tyr}$ (57%).

TABLE I

Tyrosine acceptor activity of E. coli $tRNA^{Tyr}$.

$tRNA^{Tyr}$ used	Counts per min. ^{14}C-Tyrosine Incorporated per OD_{260} unit	% of control
Native tRNA	88,000	100
Heated-CNBr	83,950	95.4
Heated+CNBr	75,000	85.2

Discussion

The basic requirements in any studies involving modification of tRNA are (i) the reaction should be selective, (ii) the reaction should be preferably quantitative and (iii) the reaction should not result in the breakdown of phosphodiester linkages. The work described in this report meets there requirements and a summary of the findings is as follows: (1) The two S residues present in E. coli $tRNA^{Tyr}$ can be quantitatively modified to U. (2) Detailed analysis of oligonucleotides produced by degradation with T_1-RNase show that the modification reaction was highly selective, the only other nucleoside modified was G^*, a part of the anticodon sequence G^*-U-A- in this tRNA. (3) The modified tRNA retains its aminoacid acceptor activity and its ability to participate in protein synthesis. (4) Studies to be reported in detail (Walker and RajBhandary, manuscript in preparation for J. Biol. Chem.) also show that the modification reaction does not result in any appreciable change in (i) the K_m for the tRNA or the specificity in the aminoaylation reaction, (ii)

the thermal denaturation profile and (iii) recognition by E. coli tRNA pyrophosphorylase. Similarly, modification of E. coli $tRNA^{fMet}$ does not affect the overall functions of this tRNA including aminoacid acceptance, recognition by E. coli methionyl tRNA transformylase and ability to initiate protein synthesis.

The modification reaction described here yields a modified tRNA which is a potential substrate for the enzyme involved in the biosynthesis of S in tRNA. Use of this modified and well characterized tRNA of known sequence would be expected to facilitate the isolation and study of the thiolating enzyme (Lipsett and Peterkofsky, 1967; Hayward and Weiss, 1967).

A possible variation of the modification reaction is conversion of S to cytidine (C) instead of U as in this report (unpublished). It is worth noting that the S common to all E. coli tRNA's (nucleoside number 8 from the 5' end) is replaced by U in yeast or rat liver tRNA's (Madison, 1968; Staehelin, et al., 1968). Whether this particular position of a tRNA needs to be occupied by U or a derivative of U is now being directly examined by modification of S to C and a study of such modification on the properties of the tRNA.

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