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The involvement of the anticodon adjacent modified nucleoside N-[9-(β-D-ribofuranosyl) purine-6-ylcarbamoyl]-threonine in the biological function of E.coli $tRNA^{ile}$.

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ABSTRACT

$tRNA^{ile}$ was isolated from E. coli Cp 79 (leu^-, arg^-, thr^-, his^-, $thiamin^-$, RC^{rel}) which had been grown on a sub-optimal concentration of thr and was found to contain an average of 50% less N-[9-(β-D-ribofuranosyl)-purin-6-ylcarbamoyl]threonine, t^6Ado, than $tRNA^{ile}$ from cells grown on an optimum concentration of thr and containing a normal complement of t^6Ado. The two tRNA's were identical in their ability to be aminoacylated, to accept the 3'-terminal dinucleotide, and to form an ile-$tRNA^{ile}$-Tu-GTP complex. In contrast, the t^6Ado-deficient-tRNA was significantly less efficient in binding to ribosomes compared to the normal tRNA. This difference was seen in the binding of deacylated tRNA and in the nonenzymatic and enzymatic binding of ile-tRNA, all in response to poly AUC. The t^6Ado-deficient ile-tRNA demonstrated no binding at Mg^{2+} concentrations ≤ 10 mM, while the normal ile-tRNA bound at low Mg^{2+} concentrations. Tetracycline had the same effect on the normal as on the t^6Ado-deficient ile-tRNA binding. As a control, the binding of phe-tRNA (which does not contain t^6Ado) from normal and thr-starved cells in response to poly U was identical. It was concluded that t^6Ado is required for proper codon-anticodon interaction.

INTRODUCTION

In almost all the tRNA primary sequences determined so far, the residue at the 3'-position adjacent to the anticodon is a modified purine nucleoside.[1] The *in vitro* effects of alteration or deletion of the 3'-anticodon adjacent nucleosides have been studied mainly for those tRNA's which are stimulated by uridine-beginning codons. The effects of removal or modification of the "Y" base in $tRNA^{phe}_{yeast}$ [2,3] or $tRNA^{phe}_{wheat\ germ}$ [4], or of the isopentenyl moiety in suppressor $tRNA^{tyr}_{E.\ coli}$ [5], $tRNA^{phe}_{E.\ coli}$ [6], or $tRNA^{ser}_{E.\ coli}$ [7] has been reported. In each case the modification resulted in altered or decreased ribosomal binding, but had no effect on aminoacylation.

tRNA's which recognize codons starting with adenosine have been found to contain the threonine substituted adenosine, N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine, t^6Ado, adjacent to the 3'-end of the anticodon[8,9]. The structure of this unusual nucleoside had been determined earlier, and its biosynthesis is now understood[10-15]. However, few studies have been published

on the direct involvement of t^6Ado in tRNA function. In studies of ribosome free trinucleotide binding to initiator $tRNA^{fMet}$, Hogenauer, et al.[16], found that the association constant for AUG binding to yeast $tRNA^{fMet}$, which contains t^6Ado, was twice that of E. coli $tRNA^{fMet}$, which has Ado instead of t^6Ado next to the anticodon. The enhanced binding of the former was attributed to the presence of the modified nucleoside. Freier and Tinoco[17] in a later report on these initiators found the same relative difference in association constants. In the presence of ribosomes, Dube, et al.[18], showed that both AUG and GUG are read by E. coli $tRNA^{fMet}$, whereas Stewart, et al.[19], found that yeast $tRNA^{fMet}$ read only AUG.

We have previously reported the decreased ribosomal binding efficiency of [^{14}C]-ile-tRNA containing a less than normal amount of t^6Ado[20]. In this communication we report the results of a more complete comparison of these normal and t^6Ado-deficient tRNA's isolated from E. coli Cp79[21].

METHODS

Preparation of Normal and t^6Ado-deficient tRNA.

The t^6Ado content of each tRNA preparation was determined as previously described[22]. E. coli Cp79 (leu^-, arg^-, thr^-, his^-, $thiamin^-$, RC^{rel})[21] was grown in a standard minimal media with 5% glucose as carbon source supplemented with 20 μg/ml of leu, arg, and his, with 2 μg/ml thiamin, and with either 2 or 20 μg/ml thr (which yielded the t^6Ado-deficient or normal tRNA respectively). The cells were grown with vigorous agitation, harvested in late-log, and washed with 0.5% NaCl/0.5% KCl. tRNA was prepared by the method of Avital and Elson[23]. It was then subjected to Sephadex G-100 chromatography[24] to remove any traces of rRNA or 5S-RNA. This material was used for the preparation of [^{14}C]-phe-tRNA and tRNApCp[^{3}H]Cp[^{3}H]A.

Purification of $tRNA^{ile}$.

The heterogeneous tRNA prepared as above (normal or t^6Ado-deficient, 10,000 $A_{260\ nm}$ units each) was fractionated by chromatography on RPC-2[25], using a 5 x 200 cm column eluted with a gradient consisting of 10 liters 0.1M NaOAc, pH 4.5, 10mM $MgSO_4$, 10mM 2-mercaptoethanol, and 0.2M NaCl; and 10 liters of the same buffer containing 0.7M NaCl. The ile-accepting tRNA, which was eluted as a broad peak between 0.35 - 0.42M NaCl, was concentrated ten-fold under reduced pressure at $< 40^o$ and concentrated by ethanol precipitation. This material which was 3-5 fold enriched in ile acceptor capacity (ile acceptance, 280-410 pmoles/$A_{260\ nm}$ unit), was used for the preparation of [^{14}C]-ile-tRNA as described below. These partially purified normal and t^6Ado-deficient tRNA's were further purified by BD-cellulose

chromatography as described by Wimmer, et al[26]. The tRNA was first charged with [^{14}C]-ile in a reaction mixture containing 100mM 3,3-dimethyl-glutaric acid buffer, pH 6.9, 10mM MgOAc, 10mM KCl 4mM ATP, 50 μM [^{14}C]-ile (10 μC/μmole), 165 mg unfractionated E. coli aminoacyl-tRNA synthetase[27], and either 750 $A_{260\ nm}$ units normal tRNA or 598 $A_{260\ nm}$ units t^6Ado-deficient tRNA. Complete charging was achieved in 20 min at 37°. The [^{14}C]-ile-tRNA was extracted with phenol and precipitated with ethanol. The recovered (100%) [^{14}C]-ile-tRNA was phenoxyacetylated by the standard method[26] to give [^{14}C]-ile-tRNA which was 83-86% derivatized. The N-phenoxyacetyl-[^{14}C]-ile-tRNA (571 $A_{260\ nm}$ units normal or 590 $A_{260\ nm}$ units t^6Ado-deficient tRNA), dissolved in 100 ml of equilibrating buffer, was loaded onto a BD-cellulose column (2 x 100 cm, 100-200 mesh) previously equilibrated with 1.0M NaCl, 10mM NaOAc, pH 5.5, 10mM $MgSO_4$, 10mM 2-mercaptoethanol, and 8% ethanol at 4°C. The column was washed with the same buffer containing 15% ethanol to elute uncharged tRNA and underivatized [^{14}C]-ile-tRNA. The N-phenoxyacetyl-[^{14}C]-ile-tRNA was eluted with the same buffer containing 30% ethanol. The tRNA had a specific activity of 1330-1360 pmoles [^{14}C]-ile per $A_{260\ nm}$ unit (89-92% pure). After concentration by ethanol precipitation, the N-phenoxyacetyl-ile was removed by incubation at 37° for 1 hr, and then 60° for 5 min in 1.8M Tris·acetate, pH 8.0, 20mM $Mg(OAc)_2$, 10mM 2-mercaptoethanol. The material recovered after ethanol precipitation contained negligible trichloroacetic acid insoluble radioactivity and was used for the preparation of normal or t^6Ado-deficient [^{14}C]-ile-$tRNA^{ile}$ and ile-$tRNA^{ile}$.

Preparation of Aminoacyl-tRNA-synthetases.

Aminoacyl-tRNA synthetases which were used for the determination of Ki values (Table I) were prepared from E. coli A-19 (General Biochemicals) by a modification of the method of Muench and Berg[28]. 30 g of cells were dispersed in 60 ml of buffer (10mM Tris·HCl, pH 7.4, 10mM $Mg(OAc)_2$, 1mM EDTA, and 1mM glutathione and broken in a French pressure cell at 10,000 to 12,000 psi. The broken cell suspension was diluted with 2 volumes of buffer and centrifuged for 30 min at 30,000 Xg and then for 3 hours at 95,000 Xg. The supernatant was subjected to liquid polymer phase fractionation as previously described[29]. The lower phase obtained after $(NH_4)_2SO_4$ precipitation was dialized against 10mM phosphate buffer, pH 7.4, containing 1mM glutathione and chromatographed on DEAE-cellulose[28]. The enzyme was stored in 50% glycerol. Immediately before use the preparation was freed of glycerol by passage through a column of Sephadex G-25 in 10mM cacodylate buffer, pH 7.5, 10mM MgOAc, 2mM 2-mercaptoethanol, 0.1mM EDTA.

Preparation of E. coli B tRNA Nucleotidyltransferase.

The enzyme was prepared as previously described[30] through the hydroxylapatite column step.

Preparation of Ribosomes.

Ribosomes were prepared from late log phase E. coli Q-13 by either the method of Philipps[24] or Chen and Ofengand[31]. The two preparations gave essentially the same results in all binding experiments.

Elongation factors Tu-Ts and purified Tu factor prepared by the method of Weissbach, et al[32], were kindly supplied by Dr. D. L. Miller of the Roche Institute.

Preparation of tRNApCp[^{3}H]Cp[^{3}H]A.

The heterogeneous normal or t^{6}Ado-deficient tRNA after digestion with venom phosphodiesterase to remove the 3'-terminal pCpA[33] was incubated with [^{3}H]-ATP, [^{3}H]-CTP, and tRNA nucleotidyltransferase to reconstitute the terminal nucleotide sequence. A typical reaction mixture contained in 20 ml: 50mM glycine·NaOH, pH 9.0, 10mM MgOAc, 2mM glutathione, 750 nmoles tRNApC, 2.5 μmoles [^{3}H]-CTP (120 μCi/μmoles), 5.0 μmoles [^{3}H]-ATP (140 μCi/μmole), and 200 mg partially purified enzyme. After 40 min at 37° the tRNA was recovered by phenol extraction of the reaction mixture and ethanol precipitation of the combined aqueous phases. The tRNApCp[^{3}H]Cp[^{3}H]A was further purified by Sephadex G-100 chromatography[24]. An identical but smaller scale reaction was run in parallel using unlabeled ATP and CTP. The tRNA from this reaction was found to have >95% of the molecules with an intact pCpCpA terminus determined as previously described[33] and an ile accepting capacity identical to the heterogeneous tRNA before phosphodiesterase digestion.

Preparation of [^{14}C]-ile-tRNA, [^{14}C]-phe-tRNA, and [^{14}C]-ile-tRNA^{ile}.

The partially purified (from RPC-2) normal or t^{6}Ado-deficient tRNA's was charged with [^{14}C]-ile and 18 unlabeled amino acids (all except leu; the presence of leu prevented quantitative charging of the tRNA^{ile}), and the heterogeneous (before RPC-2) tRNA's were charged with [^{14}C]-phe and 19 unlabeled amino acids; each in a typical 10 ml reaction mixture containing 50mM cacodylate, pH 6.9, 12mM MgOAc, 10mM KCl, 1.5mM ATP, 5mM glutathione, 450 nmoles tRNA, 0.1 μmole [^{3}H]-ile (10 mCi/μmole), 0.1 μmole of the other 18 (minus leu) amino acids, and 9 mg partially purified aminoacyl tRNA synthetases. After 25 min at 25°, the tRNA was recovered as described above for the preparation of tRNApCp[^{3}H]Cp[^{3}H]A. In the case of tRNA^{ile} (from BD-cellulose) the reaction was one-fifth this size and the 18 cold amino acids were omitted.

Aminoacyl-tRNA Synthetase Assay

The 0.5 ml reaction mixture contained 50 μmoles cacodylate buffer, pH 7.4; 5 μmoles $Mg(OAc)_2$; 1 μmole ATP; 2.5 μmoles KCl; 0.75 nmoles [^{14}C]-labeled L-amino acid 0.1-0.3 μCi; 1-100 nmoles tRNA; and an amount of the aminoacyl-tRNA synthetase preparation that would give a linear rate of amino-acylation (approximately 0.02-0.10 mg). The reaction was initiated by adding the enzyme. After an appropriate incubation period to give kinetically valid results (10-20 min) at 37°, a 50 μl sample was pipetted onto a 2.3 cm Whatmann 3MM paper disk and the disk was dipped in 10% trichloroacetic acid. The disks were then successively washed for 10 min in 5% trichloroacetic acid (twice) and 50% ethanol in diethylether and then diethylether (once each). The disks were dried under an infrared light for 1 hr and counted in toluene based scintillation fluid in a liquid scintillation spectrometer.

tRNA-nucleotidyltransferase Assay

The standard assay contained in 0.1 ml: 5.0 μmoles of glycine-NaOH (pH 9.2), 1.0 μmole of Mg^{2+} acetate, 1.0 μmole of glutathione, 1-100 nmoles of venom phosphodiesterase-treated tRNA, and 0.01 to 5.0 μg of protein. For AMP incorporation 20 nmoles (0.1 μCi) of 8-[^{3}H]-ATP and tRNA pCpC were used. The assay for CMP incorporation contained 20 nmoles (0.1 μCi) of 5-[^{3}H]-CTP and either tRNA-X-C or tRNA-X. All assays were conducted under conditions which gave kinetically valid data as determined by pilot experiments. After 5 to 30 min at 37°, the reaction was stopped by addition of 2 ml of 20mM EDTA containing 0.2mM ATP, followed by 2 ml of 10% trichloracetic acid. For a control, an identical reaction mixture was precipitated at zero time. The precipitate was collected on glass fiber filters and washed with 3.5% trichloracetic acid and 95% ethanol. The filters were dried and counted in 3 ml of a toluene-based scintillator. The tRNApCpCp and tRNApC used as substrates were prepared by venom phosphodiesterase treatment[33] of the heterogeneous normal or t^6Ado-deficient E. coli Cp79 described above.

Km values for both the aminoacyl-tRNA-synthetases and tRNA-nucleotidyl-transferase were determined from Lineweaver-Burk plots.

Ribosome Binding Assays

Ribosomes were suspended in the appropriate buffer (specified in Figures 1-5) at a concentration 4 times the final concentration in the assay, aggregates were removed by centrifugation (5000 Xg for 20 min). The components of the assays are noted in the figures. All other components were preincubated for 2 min at 37° before the initiation of the reaction by the addition of tRNA. The reaction was stopped after an appropriate period by the addition of 2 ml of ice-cold buffer of the same composition in which the assay was performed and

the samples were filtered through Millipore filters[34]. The filters were washed (3x) with the same buffer, dried, and counted in toluene based scintillator in a liquid scintillation spectrometer. To determine the cpm in a known amount of tRNA in a known reaction mixture, an aliquot of the entire reaction mixture was streaked onto a dry Millipore filter, dried without washing, and counted. All results reported are mean values of duplicates.

Formation of ile-tRNA^{ile}-Tu-[^{3}H]-GTP Complexes.

The formation of Tu-GTP and ile-tRNA-Tu-GTP complexes was assayed by retention of the Tu-[^{3}H]-GTP complex on Millipore filters and the reduction in this retained radioactivity which occurs due to the passage of the ile-tRNA-Tu-[^{3}H]-GTP ternary complex through the filter[35]. Tu-GDP, the form in which Tu is prepared, and any radioactive GDP in the commercial GTP preparation were first converted to Tu-GTP and GTP, respectively, by preliminary incubation at 37°C for 10 min in a reaction mixture containing 50mM Tris·HCl, pH 7.4, 50mM NH_4Cl, 10mM $MgCl_2$, 5mM dithiothreitol, 7.5mM phosphoenolpyruvate, 100 μg of pyruvate kinase (Boehringer), 0.5mM [^{3}H]-GTP (19.5 C/mmole) and 300-400 units of purified Tu-GDP in a volume of 1 ml. ile-tRNA was added to 50 μl of the Tu-GTP mixture at 0° to make the final volume of 0.2 ml, and incubation was continued for 5 min at 0°. At the end of the reaction, 0.1 ml of the reaction mixture was immediately streaked by micropipette on a Millipore filter prewetted with washing buffer (50mM Tris·HCl pH 7.5, 50mM NH_4Cl, 10mM $MgCl_2$) under suction. The filter was washed three times with 1 ml of cold washing buffer, dissolved in 10 ml of Aquascint II (ICN Life Sciences) plus 1 ml water, and counted. The amount of ternary complex formed was computed as the difference in filter-bound radioactive GTP in the absence and presence of ile-tRNA.

RESULTS

Synthesis of tRNA with a Decreased Content of t^6Ado by a thr Requiring RC^{rel} Mutant of E. coli.

E. coli Cp79 (leu$^-$, arg$^-$, thr$^-$, his$^-$, thiamin$^-$, RC^{rel}) can synthesize RNA when starved for any of these amino acids[21]. Our first attempts to produce tRNA which contained a less than normal complement of t^6Ado involved growing E. coli Cp79 at a concentration of thr that allowed normal growth (20 μg/ml) and which resulted in a normal concentration of t^6Ado in its tRNA (~ 0.2 mole%, based on 80 nucleotides tRNA); and then, when the cells reached the mid-log stage of growth, to wash the cells in media lacking thr, resuspend them in thr-free media, and then allow them to continue to divide until growth stopped. By this method, the tRNA that was isolated contained only slightly

less t^6Ado (0.15-0.18 mole%). We then tried growing the cells on low concentrations of thr from the beginning of the culturing period, and allowing the cells to divide until growth stopped. It was found that the amount of t^6Ado in the tRNA was reduced at 10 μg/ml or lower thr, but decreasing the thr concentration below 2.0 μg/ml did not result in further decreasing the t^6Ado content of the tRNA. At all thr concentrations below 20 μg/ml, the yield of cells was decreased. The t^6Ado-deficient tRNA used in the experiments reported here was all isolated from cells grown on 2.0 μg/ml thr, and contained 0.08-0.13 mole% t^6Ado. The normal tRNA was isolated from cells grown on 20 μg/ml thr, and contained 0.21-0.25 mole% t^6Ado.

Comparison of the Biochemical Properties of Normal and t^6Ado-deficient tRNA's.

The various biochemical functions in which tRNA takes place were examined using the normal and t^6Ado-deficient tRNA preparations. These functions can be divided into two classes: those which are apparently independent of the anticodon portion of tRNA (aminoacylation, 3'-terminal nucleotide addition, and Tu-GTP-tRNA complex formulation), and those which involve the anticodon (codon-dependent binding of tRNA to ribosomes).

Interaction of Normal and t^6Ado-deficient tRNA with Aminoacyl-tRNA Synthetases and tRNA Nucleotidyltransferase.

The Km values for tRNA with 12 different amino acids as substrate using a partially purified synthetase preparation which contained all 12 of these synthetase activities were determined for both the normal and t^6Ado-deficient tRNA. The results (Table I) show that there was no significant difference in the Km values between the tRNA's which contained a reduced amount of t^6Ado and the normal tRNA's.

The normal and t^6Ado-deficient tRNA preparations were both treated with venom phosphodiesterase to remove the 3'-terminal -pCpA. To a portion of each of the two tRNA preparations the tRNApCpC sequence was completed by enzymatic addition of the last pC with the tRNA nucleotidyltransferase. The Km values for tRNA for the addition of CMP to tRNApC and for the addition of AMP to tRNApCpC were determined for both the normal and t^6Ado-deficient tRNA's. The data (Table I) show that the partial lack of the t^6Ado produced no significant effect on the ability of tRNA to interact with this enzyme.

Binding of Normal and t^6Ado-deficient Deacylated tRNA to Ribosomes.

Both the normal and deficient tRNA were labeled in the 3'-terminal dinucleotide (tRNApCpCpA) in order to ascertain the effect of t^6Ado-deficiency on the binding of deacylated tRNA to ribosomes in the presence of poly AUC and poly U. AUC codes for ile and $tRNA^{ile}$ contains t^6Ado next to its anticodon[36].

Table I. Km Values for Normal and t^6Ado-deficient tRNA for Aminoacylation and pCpA Addition.

Aminoacylation	Km for Normal tRNA	Km for t^6Ado-Deficient tRNA
A-beginning codons	($M \times 10^7$)	($M \times 10^7$)
Ile	4.5	2.9
Thr	9.0	11.0
Met	69	47
Lys	0.91	0.97
Ser	4.7	6.6
Arg	12	18
U-beginning codons		
Phe	9.1	8.0
Ser	1.9	2.7
C-beginning codons		
Leu	2.7	1.0
Arg	7.0	5.1
G-beginning codons		
Val	11	16
Gly	2.4	3.8

pCpA Addition	Normal tRNA	t^6Ado-Deficient tRNA
	($M \times 10^7$)	($M \times 10^7$)
AMP Addition to tRNApCpC	2.6	1.2
CMP Addition to tRNApC	3.0	5.1

UUU codes for phe and $tRNA^{phe}$ does not contain t^6Ado^{37}. The t^6Ado-deficient tRNA demonstrated significantly less binding than the normal tRNA in response to poly AUC (Figure 1A). In the presence of poly U this difference in binding was not observed (Figure 1B). The non-specific binding in the absence of polymer was approximately the same for the two tRNA preparations.

Binding of Normal and t^6Ado-deficient [^{14}C]-ile-tRNA to Ribosomes.

The time course of the binding of normal and t^6Ado-deficient [^{14}C]-ile to salt-washed ribosomes in response to poly AUC shows that both the apparent rate of binding and the maximum extent of binding was less for the t^6Ado-deficient [^{14}C]-ile-tRNA than for the normal [^{14}C]-ile-tRNA (Figure 2A).

The normal and t^6Ado-deficient [^{14}C]-ile-tRNA demonstrated similar polymer dependence (Figure 2B). Poly AUC gave the most efficient binding, poly AUU resulted in a less efficient but significant binding, and poly AUA did not stimulate binding. In the case of poly AUC and poly AUU the binding of the t^6Ado-deficient [^{14}C]-ile-tRNA was stimulated to a lesser extent than the binding of normal [^{14}C]-ile-tRNA.

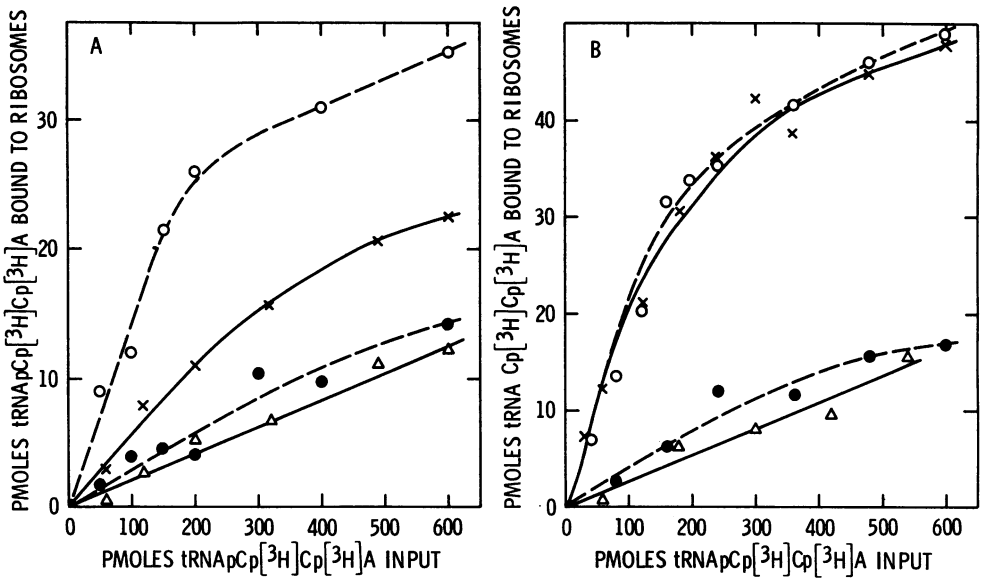

Figure 1A. The Poly AUC-Directed Binding of Normal and t^6Ado-Deficient Deacylated tRNA to Ribosomes. The 100 µl assay contained 2.0 µmoles Tris·HCl, pH 7.5, 1.0 µmoles MgOAc, 10 µmoles KCl, 5.0 µmoles NH_4Cl, 8.0 $A_{260\,nm}$ units ribosomes, 200 µg poly AUC (1:1:1), and varying concentrations of either normal tRNApCp[^{3}H]Cp[^{3}H]A (0.24 mole % t^6Ado, 360 cpm/pmole) or t^6Ado-deficient tRNApCp[^{3}H]Cp[^{3}H]A (0.11 mole % t^6Ado, 280 cpm/pmole). Each assay mixture was preincubated for 2.0 min at 37° before the binding was initiated by the addition of the tRNApCp[^{3}H]Cp[^{3}H]A. Incubation was continued for an additional 15 min (time of maximum binding) and then terminated by filtering and washing as described in METHODS. Normal tRNApCp[^{3}H]Cp[^{3}H]A plus poly AUC (O---O) and minus poly AUC (●---●); t^6Ado-deficient tRNApCp[^{3}H]Cp[^{3}H]A plus poly AUC (X——X) and minus poly AUC (Δ——Δ).

Figure 1B. The Poly U-Directed Binding of Normal or t^6Ado-Deficient Deacylated tRNA to Ribosomes. The assay mixtures and procedures were similar to those used in Figure 1A except that the poly AUC was replaced by 20 µg poly U and the incubation time was 10 min. Normal tRNApCp[^{3}H]Cp[^{3}H]A plus poly U (O---O) and minus poly U (●---●); t^6Ado-deficient tRNApCp[^{3}H]Cp[^{3}H]A plus poly U (X——X) and minus poly U (Δ——Δ).

The tRNA dependency of the normal and t^6Ado-deficient [^{14}C]-ile-tRNA (Figure 3A) shows that at "low" tRNA concentrations, in response to poly AUC or poly AUU, the two tRNA's demonstrated the same binding efficiency. At "high" tRNA concentrations, no additional t^6Ado-deficient [^{14}C]-ile-tRNA bound to the ribosomes, but additional normal [^{14}C]-ile-tRNA bound, which resulted in a greater net binding of the latter at saturating tRNA concentrations.

When the binding of purified normal and t^6Ado-deficient [^{14}C]-ile-tRNA^{ile} was compared, the deficient tRNA demonstrated less efficient binding at all concentrations of tRNA (Figure 3B). In contrast, normal and t^6Ado-deficient [^{14}C]-phe-tRNA were identical in their binding efficiency at all tRNA concentrations (Figure 3C).

At Mg^{2+} concentrations less than 10mM the t^6Ado-deficient [^{14}C]-ile-tRNA did not bind to ribosomes (Figure 4A). In comparison, the normal [^{14}C]-ile-tRNA demonstrated significant binding at 4mM Mg^{2+}. The t^6Ado-deficient tRNA

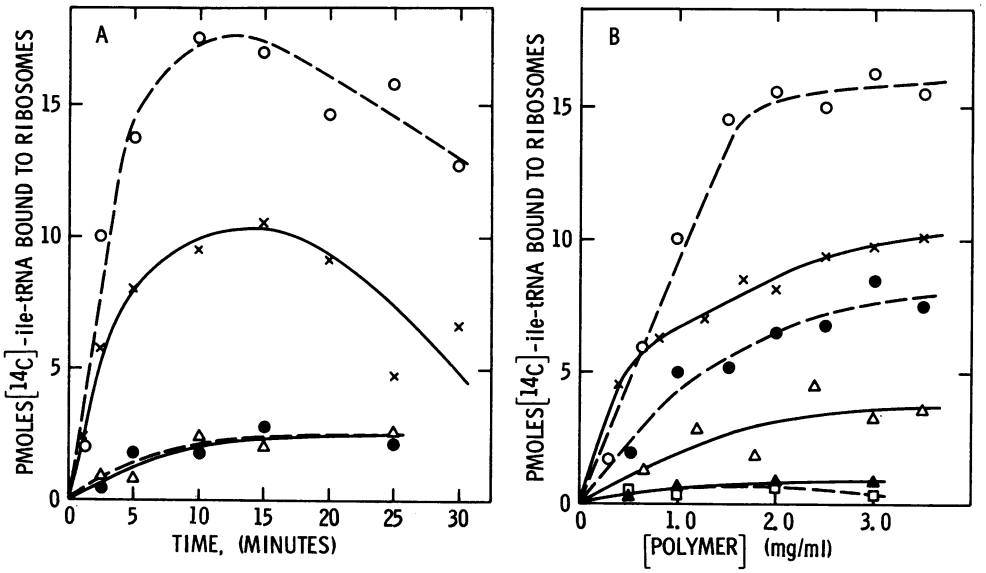

Figure 2A. Time Course of Poly AUC-Directed Non-Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-ile-tRNA to Ribosomes. The 100 µl assay contained 2.0 µmoles Tris·HCl, pH 7.5, 2.0 µmoles MgOAc, 10 µmoles KCl, 5.0 µmoles NH_4Cl, 10 A_{260} nm units ribosomes, 200 µg poly AUC (1:1:1), and 0.2 nmoles of either normal [^{14}C]-ile-tRNA (0.25 mole % t^6Ado, 367 cpm/pmole) or t^6Ado-deficient [^{14}C]-ile-tRNA (0.08 mole % t^6Ado, 359 cpm/pmole). The assay mixtures were processed as described in Figure 1A. Normal [^{14}C]-ile-tRNA plus poly AUC (O---O) and minus poly AUC (●---●); t^6Ado-deficient [^{14}C]-ile-tRNA plus poly AUC (X—X) and minus poly AUC (Δ—Δ).

Figure 2B. Polymer Dependence of the Non-Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-ile-tRNA to Ribosomes. The assay mixtures and procedures were similar to those used in Figure 2A except that varying concentrations of either poly AUC (1:1:1), poly AUU [poly AU (1:2)], or poly AUA [poly AU (2.2:1)] were used. The normal [^{14}C]-ile-tRNA (0.23 mole % t^6Ado) and the t^6Ado-deficient [^{14}C]-ile-tRNA (0.13 mole % t^6Ado) had specific activities of 280 and 318 cpm/pmole, respectively. Normal tRNA plus poly AUC (O---O), plus poly AUU (●---●), or plus poly AUA (▲—▲); t^6Ado-deficient tRNA plus poly AUC (X—X), plus poly AUU (Δ—Δ), or plus poly AUA (□---□).

demonstrated less efficient binding at all Mg^{2+} concentrations tested, but the greatest difference was seen at 10mM Mg^{2+}. In the presence of 50 μM tetracycline, these same differences were observed. The degree of inhibition of binding at increasing tetracycline concentrations was examined for the normal

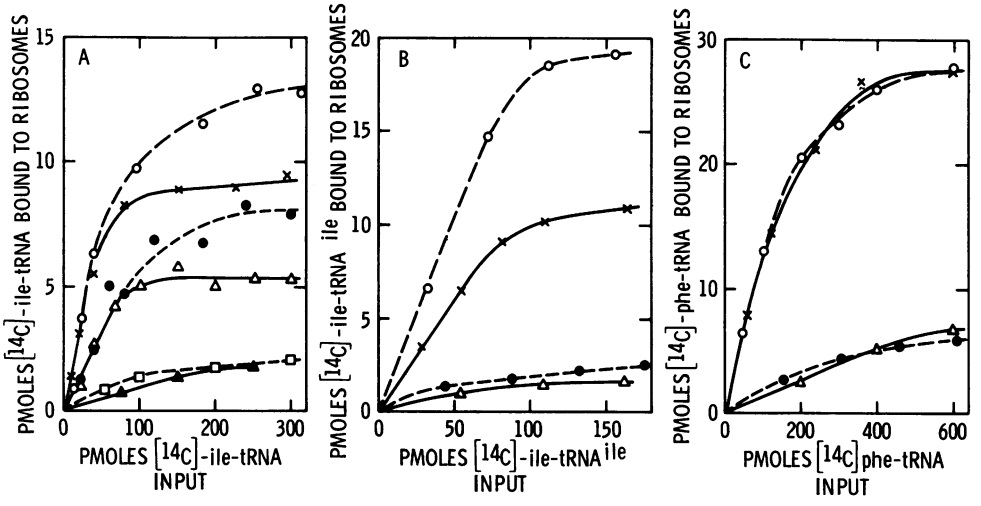

Figure 3A. tRNA Dependence of the Poly AUC and Poly AUU-Directed Non-Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-ile-tRNA to Ribosomes. The assay mixtures and procedures were similar to those used in Figure 2A except that poly AUU replaced poly AUC in some cases and varying concentrations of either normal [^{14}C]-ile-tRNA (0.25 mole % t^6Ado, 367 cpm/pmole) or t^6Ado-deficient [^{14}C]-ile-tRNA (0.08 mole % t^6Ado, 359 cpm/pmole) were used. Normal [^{14}C]-ile-tRNA plus poly AUC (O---O), plus poly AUU (●---●), and minus polymer (□---□); t^6Ado-deficient [^{14}C]-ile-tRNA plus poly AUC (X——X), plus poly AUU (Δ——Δ), and minus polymer (▲—▲).

Figure 3B. tRNA Dependence of the poly AUC-Directed Non-Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-ile-tRNA^{ile} to Ribosomes. The assay mixtures and procedures were similar to those used in Figure 2A except that 12 A_{260} nm units of ribosomes, 1.0 μmole MgOAc, and varying concentrations of either normal [^{14}C]-ile-tRNA^{ile} (0.24 mole % t^6Ado, 350 cpm/pmole) or t^6Ado-deficient [^{14}C]-ile-tRNA^{ile} (0.08 mole % t^6Ado, 320 cpm/pmole) were used. Normal [^{14}C]-ile-tRNA^{ile} plus poly AUC (O---O) and minus polymer (●---●); t^6Ado-deficient [^{14}C]-ile-tRNA^{ile} plus poly AUC (X——X) and minus polymer (Δ——Δ).

Figure 3C. tRNA Dependence of the Poly U-Directed Non-Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-phe-tRNA to Ribosomes. The assay mixtures and procedures were similar to those used in Figure 2A except that 8.0 A_{260} nm units of ribosomes, 20 μg poly U, and varying concentrations of either normal [^{14}C]-phe-tRNA (0.24 mole % t^6Ado, 160 cpm/pmole) or t^6Ado-deficient [^{14}C]-phe-tRNA (0.11 mole % t^6Ado, 210 cpm/pmole) were used. Normal [^{14}C]-phe-tRNA plus poly U (O---O) and minus polymer (●---●); t^6Ado-deficient [^{14}C]-phe-tRNA plus poly U (X——X) and minus polymer (Δ——Δ).

and t^6Ado-deficient [^{14}C]-ile-tRNA (Figure 4B). No difference was seen in the action of tetracycline between the two tRNA preparations; the binding of both was 50% inhibited at about 0.1mM drug (Figure 4B).

When the ribosomal binding was examined in the presence of Tu-Ts and GTP (Figure 5A), the decreased binding efficiency of the t^6Ado-deficient tRNA compared to the normal-tRNA was still observed. In contrast, the formation of the ile-tRNA-Tu-GTP complex showed the same tRNA dependence whether normal or t^6Ado-deficient ile-tRNA was used (Figure 5B).

DISCUSSION

The tRNA from thr-starved Cp79 would be expected to partially lack the thr moiety of t^6Ado. Elkins and Keller[15] have isolated t^6Ado-deficient tRNA from Cp79 and used it as a substrate for the enzymic synthesis of t^6Ado. The nature of this enzymic reaction suggests that the t^6Ado-deficient tRNA contains

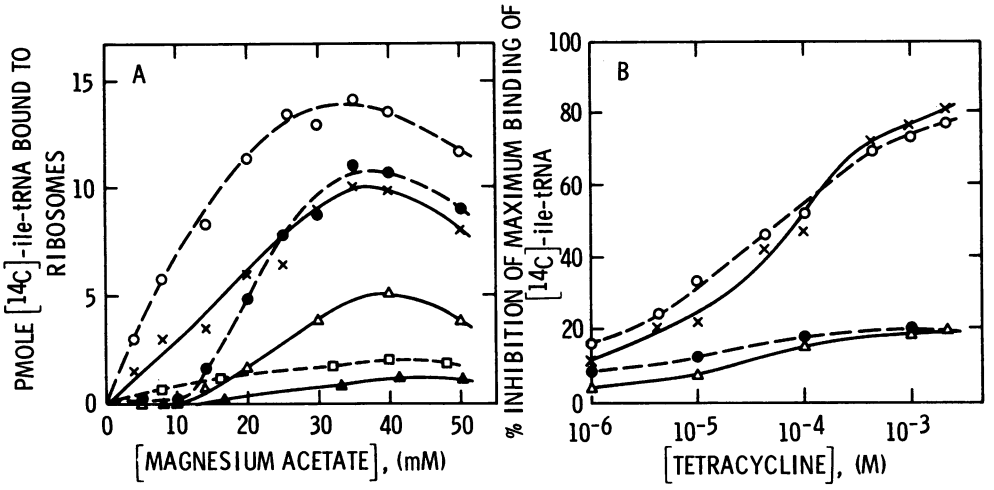

Figure 4A. Effect of [Mg^{2+}] on the Poly AUC-Directed Non-Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-ile-tRNA to Ribosomes in the Presence and Absence of Tetracycline. The assay mixtures and procedures were similar to those used in Figure 2A except that 8.0 A_{260} nm units of ribosomes, 0.2 nmoles of either normal [^{14}C]-ile-tRNA (0.21 mole % t^6Ado, 409 cpm/pmole) or t^6Ado-deficient [^{14}C]-ile-tRNA (0.09 mole % t^6Ado, 367 cpm/pmole), and where indicated 50 μM tetracycline, were used. Normal [^{14}C]-ile-tRNA minus tetracycline (O---O), plus tetracycline (X——X), and minus poly AUC either with or without drug (□---□); t^6Ado-deficient [^{14}C]-ile-tRNA minus tetracycline (●---●), plus tetracycline (Δ——Δ), and minus poly AUC either with or without drug (▲——▲).
Figure 4B. Effect of [Tetracycline] on the Poly AUC-Directed Non-Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-ile-tRNA to Ribosomes. The assay mixtures and procedures were identical to those used in Figure 2A with the addition of varying concentrations of tetracycline. Normal [^{14}C]-ile-tRNA plus tetracycline (O---O) and minus tetracycline (●---●); t^6-Ado-deficient [^{14}C]-ile-tRNA plus tetracycline (X——X) and minus tetracycline (Δ——Δ).

Ado in place of t^6Ado adjacent to the 3'-end of the anticodon. Elkins and Keller[15] suggested that, in the absence of thr, gly would replace the thr to yield N-[9-(β-D-ribofuranosyl)-purin-6-ylcarbamoyl]glycine, g^6Ado, although they did not quantitate this nucleoside in their Cp79 tRNA preparations. The isolation and characterization of g^6Ado from yeast tRNA has been described[38]. We have also found g^6Ado in E. coli tRNA and have noted that the t^6Ado-deficient tRNA contains no more g^6Ado than the normal tRNA preparations from Cp79[39]. On the basis of the available data, we assume that Ado exists in place of t^6Ado in the t^6Ado-deficient tRNA.

A report has appeared[40] describing the hypermodified base, 3-(3-amino-3-carboxypropyl)uridine, 4abu^{3}Udo, isolated from tRNA ^{phe} E. coli B. The possibility was suggested[40] that this nucleoside could be phenoxyacetylated and that the derivatization of this nucleoside or others similar to it could account for

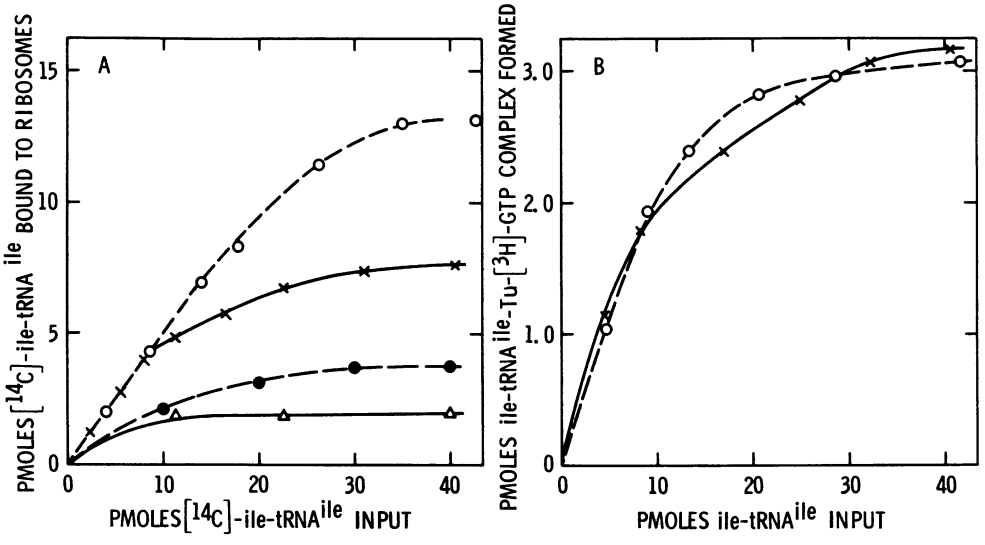

Figure 5A. tRNA Dependence of Poly AUC-Directed Enzymatic Binding of Normal and t^6Ado-Deficient [^{14}C]-ile-tRNA^{ile} to Ribosomes. The 100 µl assay contained 2.0 µmoles Tris·HCl, pH 7.5, 1.6 µmoles MgOAc, 10 µmoles KCl, 5.0 µmoles NH_4Cl, 0.5 µmoles dithiothreitol, 0.2 nmoles GTP, 8.0 $A_{260\ nm}$ units ribosomes, 10 µg purified T-factor, and varying concentrations of either normal or t^6Ado-deficient [^{14}C]-ile-tRNA^{ile} (same as used in Figure 3B). The assays were processed as described in Figure 1A. Normal [^{14}C]-ile-RNA^{ile} plus T-factor (O---O) and minus T-factor (●—●); t^6Ado-deficient [^{14}C]-ile-tRNA^{ile} plus T-factor (X—X) and minus T-factor (Δ—Δ).

Figure 5B. tRNA Dependence of ile-tRNA^{ile}-Tu-[^{3}H]-GTP Complex Formation. The assay was performed as described in METHODS using the normal (O---O) and t^6A-deficient (X—X) tRNA described in Figure 3B but charged with unlabeled ile.

the retention of several uncharged E. coli tRNA's on benzoylated DEAE-cellulose[41]. We assume that 4abu^{3}Udo is present in strain Cp79. For this reason we examined our tRNA^{ile} preparations, which had been isolated by phenoxyacetylation procedures, for aminoacylation with those amino acids whose tRNA's were found to bind to benzoylated DEAE-cellulose after derivatization of uncharged tRNA; namely, tyr, thr, ile, cys, and asn[41]. Only ile-acceptance was found[39]. Another concern was that the 4abu^{3}Udo was derivatized during the purification of the normal- and t^{6}Ado-deficient tRNA^{ile}, and might therefore change the biological properties of the tRNA's. To test this possibility we phenoxyacetylated the uncharged normal- and t^{6}Ado-deficient tRNA^{ile} preparations and examined their chromatographic properties on benzoylated DEAE-cellulose. For both the normal and t^{6}Ado-deficient tRNA's, all of the ile-accepting activity was eluted from this absorbent with 1.0M NaCl whether or not it had been subjected to the phenoxyacetylation reaction[39], suggesting no derivatization of 4abu^{3}Udo. One possible explanation for the difference between our results and those of Friedman[41] is the shorter incubation time used by us (10 min) compared to that used by Friedman (60 min).

The results presented in this report show that the lack of the modification on the adenosine adjacent to the 3'-end of the anticodon in tRNA$^{ile}_{E.\ coli\ Cp79}$ specifically diminishes the ribosomal binding efficiency of this tRNA. It has no significant effect on those processes that presumably do not involve the anticodon, namely, aminoacylation, terminal pCpA addition, and ternary complex formation. This effect on the ribosomal binding activity is consistent with the effects seen after the removal or modification of other codon-adjacent nucleosides in other tRNA's[27]. The crystal structure of the base of t^{6}Ado[42] revealed that the conformation of the molecule and an internal intramolecular hydrogen bond completely blocks the N^6-H and N^1 of the adenine moiety from taking part in Watson-Crick base pairing. Parthasarathy, et al[42], point out that the inability to base pair according to the Watson-Crick scheme appears as a common structural feature in all modified bases adjacent to the 3'-end of anticodons. They further suggest this feature may prevent misreading of the anticodons and/or promote a preferred single stranded conformation of the anticodon loops. If the lack of t^{6}Ado results in a change in the conformation of the anticodon loop of tRNA$^{ile}_{E.\ coli\ Cp79}$, then this putative conformational change may be responsible in part for the decreased ribosomal binding efficiency of the t^{6}Ado-deficient tRNA. It has previously been shown by NMR studies in solution that the

conformations of the oligonucleotide pairs ApA, Api^{6}A and 2'-OMeGpApApYpApψ, 2'-OMeGpApAp-pApψ (missing the Y-base) are significantly different[43,44] mainly due to the hydrophopic influence of the modified base. Thus maintenance of a correct anticodon loop conformation by stacking interactions with neighboring bases may be a function of the modified base.

A second possible influence could involve the thr side chain of t^{6}Ado interacting specifically with some site on the ribosome to facilitate binding. The data presented in Figure 4A shows that t^{6}Ado-deficient ile-tRNA demonstrated no apparent binding to ribosomes below 10 mM Mg^{2+}, whereas the normal ile-tRNA bound significantly at 4 mM Mg^{2+}. Since 43% of the tRNA molecules in the t^{6}Ado-deficient ile-tRNA preparation used in this experiment contained the t^{6}Ado moiety, the complete lack of binding at low Mg^{2+} concentrations was unexpected. We have repeated this experiment with three different tRNA preparations and two different ribosome preparations with essentially the same results. Although we are unable to rationalize the complete lack of binding below 10 mM Mg^{2+}, the data is nevertheless suggestive that t^{6}Ado may be a Mg^{2+} binding site. Thus the thr side chain and some specific ribosomal site may serve as ligands for Mg^{2+} in stabilizing the codon-anticodon interactions. It is difficult to conceive of how the many structurally and chemically different entities (t^{6}Ado, i^{6}Ado, i^{6}ms^{2}Ado, Y-base, etc.) could all interact specifically with essentially the same site on the ribosome, except that they almost invariably contain functional groups which are capable of binding Mg^{2+}. In fact, the double bond of the isoprene side chain in i^{6}Ado has previously been suggested as a Mg^{2+} binding site[45]. It is probable that the anticodon adjacent modified nucleosides play a necessary but complex role in codon-anticodon interactions, including those mentioned, namely, blockage of Watson-Crick base pairing, stabilizing functional anticodon loop conformations and serving as Mg^{2+} binding ligands for Mg^{2+} bridged tRNA-ribosome interactions. Mg^{2+} binding may also be required for "correct" anticodon loop conformations.

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