N-(PURIN-6-YLCARBAMOYL)THREONINE: BIOSYNTHESIS IN VITRO IN TRANSFER RNA BY AN ENZYME PURIFIED FROM *ESCHERICHIA COLI*

Ann KÖRNER and Dieter SÖLL
Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, USA

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1. Introduction

The modified nucleoside *N*-(purin-6-ylcarbamoyl)-threonine (t^6A), or derivatives of this nucleoside, is found adjacent to the 3′-end of the anticodon in tRNAs responding to codons begining with adenine, both in prokaryotic and eukaryotic organisms [1–4]. While the role of this modified nucleoside in the biological functions of tRNA is unknown, in vitro experiments [5] suggest this modified nucleoside may be responsible for correct recognition of codons that begin with adenine, or for correct orientation of the tRNA on the ribosome.

This modified nucleoside belongs to the class of hypermodified nucleosides, more complex modified nucleosides [6,7] bearing a reactive side chain. Consequently, its biosynthesis may involve more than one step. In vivo studies on the biosynthesis of t^6A in the rat [8] and *E. coli* [8,9] showed that the threonine moiety of t^6A is derived from free threonine. With this knowledge we set out to study the in vitro incorporation of radioactive threonine into t^6A containing tRNA. For these studies a tRNA substrate is required that normally contains t^6A but which can be produced lacking this modification. Recently, a defective λφ80 hybrid transducing phage that carries the *E. coli* genes for a $tRNA^{Thr}$, a $tRNA^{Tyr}$ and a $tRNA^{Gly}$ species has been described [10]. When a lysogen of this phage is induced, the tRNAs coded by the phage genes are produced in increased amounts but are often undermodified (see refs. [10, 11]). Since $tRNA^{Thr}$ contains t^6A [1–4] we used the tRNA from an induced *E. coli* lysogen of this phage as a substrate for detection of the enzyme(s) responsible for t^6A synthesis. In this communication we describe the detection of this enzyme from *E. coli*, some characteristics of the modification reaction and the tRNA substrate which is modified.

2. Materials and methods

2.1. *General*

[^{3}H]-L-threonine (specific activity 2.38 Ci/mmole), [^{14}C]-L-threonine (specific activity 186 mCi/mmole), and carrier free [^{32}P] orthophosphate were obtained commercially. Marker standards t^6Ap and t^6A were the generous gifts of Dr. S. Nishimura and Dr. G.B. Chheda, respectively. Unfractionated tRNA was prepared as described previously [12].

2.2. *Bacterial strains*

A threonine auxotroph of *E. coli* Hfr K12 *cys*$^-$ *met*$^-$ *rel*$^-$ (λ) [13] was made by screening colonies from a culture mutagenised with nitrosoguanidine. After curing this strain of λ [14] and mating with *E. coli* F$^-$ BF266 trp_{A36} [10] to introduce the trp_{A36} mutation a recombinant auxotrophic for cysteine, tryptophan and threonine was selected. This strain (KL358, *E. coli* K12 *cys*$^-$ *thr*$^-$ trp_{A36} *rel*$^-$) was lysogenised with the lysis defective hybrid λ-h80 *glyT*su^+_{36} [10] kindly given by Dr. J. Carbon. The *glyT*su^+_{36} gene on the phage suppresses the trp_{A36} mutation so that the phenotype of the lysogen (KL359) is *trp*$^+$.

For preparation of tRNA, KL359 cells were grown

at 34°C in M9 minimal medium, supplemented with 40 μg/ml cysteine and threonine. The phage was induced by raising the temperature to 42°C for 10 min; subsequent growth ($4\frac{1}{2}$ hr) was at 38°C. For the preparation of [^{32}P] phosphate labelled tRNA, a low phosphate minimal medium [15] containing 0.5 mM sodium glycerophosphate was used and [^{32}P] orthophosphate was added 10 min after induction.

2.3. *Preparation of threonine incorporating enzyme activity*

All steps were carried out at 4°C. Frozen *E. coli* (strain MRE600) cells (20 g) were ground with alumina and extracted with 60 ml of buffer A consisting of 0.2 M Tris–HCl (pH 7.6)–0.01 M magnesium chloride–0.05 M potassium chloride–0.01 M 2-mercaptoethanol–10% (v/v) glycerol. After centrifugation of the extract at 100 000 *g* for 2 hr the supernatant was loaded onto a DEAE-cellulose column (2 × 20 cm) equilibrated with buffer A. The column was washed with 250 ml of buffer A containing 0.2 M NaCl. Then the desired enzyme activity was eluted with buffer A containing 0.5 M NaCl. The eluate was dialyzed against buffer A containing 50% glycerol. The enzyme preparation (final volume 20 ml) contained 6.2 mg protein/ml and was stored at −20°C. Under these conditions the activity was stable for at least 8 weeks.

2.4. *Assay for t^6A formation*

The assay was devised to allow visualisation and quantitation of t^6Ap formation. Each assay (160 μl) contained per ml: 12 μmoles Tris–HCl (pH 7.6)–6 μmoles $MgCl_2$–31 μmoles KCl–6 μmoles 2-mercaptoethanol–6% (v/v) glycerol–0.6 μmoles [^{3}H] threonine or 0.8 μmoles [^{14}C] threonine–150 000 cpm (1 A_{260} unit) of ^{32}P-labelled tRNA (from induced KL359)–either 24 A_{260} units of unfractionated tRNA or 6 A_{260} units of purified $tRNA^{Thr}$ with 18 A_{260} units of rRNA as carrier. After incubation at 37°C for the specified time (see fig. 2), the reaction mixture was extracted with phenol and extensively dialyzed against glass distilled water. The RNA was digested with ribonuclease T2 and the hydrolysate subjected to two-dimensional thin layer chromatography. Recovery of RNA was between 25 and 30%. After autoradiography the spot corresponding to t^6Ap was scraped from the plate and counted on open channels for ^{3}H and ^{32}P. The spillover from ^{32}P into the ^{3}H channel was 5% of the ^{32}P counts. All radioactivity in the tritium channel in excess of this represented incorporation of threonine into t^6Ap.

2.5. *Analysis and identification of t^6A*

The analysis at the nucleotide level was performed as described earlier [15] by two-dimensional thin-layer cochromatography of ^{3}H/^{32}P labelled t^6Ap from a ribonuclease T2 digest of the tRNA with an authentic standard. The solvent systems used were: I, isobutyric acid–0.5 M ammonium hydroxide (5:3, v/v) and II, isopropyl alcohol–conc. hydrochloric acid–water (70:15:15, v/v). The analysis at the nucleoside level was performed by treating [^{14}C]t^6Ap with alkaline phosphatase, followed by descending paper chromatography in solvents I and II, and paper electrophoresis at pH 7.0 with similarly treated authentic samples. [^{14}C] Threonine was detected after treatment of [^{14}C]t^6A with 0.5 M NaOH for 30 min at 100°C followed by paper electrophoresis at pH 7.0 [8].

2.6. *Aminoacylation of tRNA*

This was performed as described earlier [12] with a mixture of *E. coli* K12 aminoacyl-tRNA synthetases freed from tRNA by DEAE-cellulose chromatography.

3. Results

3.1. *Production of undermodified $tRNA^{Thr}$ following phage induction*

For the detection of the threonine incorporating enzyme activity, unfractionated tRNA obtained after induction of the lysogen *E. coli* KL359, was used. This tRNA was compared to that produced in the uninduced lysogen by reversed phase cochromatography of the two tRNAs charged with [^{3}H] threonine and [^{14}C] threonine, respectively. The results, indicating a new form of $tRNA^{Thr}$ produced in induced cells, were similar to those obtained by Squires et al. [10].

The additional tRNA peak is the undermodified form of $tRNA^{Thr}$ produced by the phage. To obtain large amounts of this $tRNA^{Thr}$ species we purified $tRNA^{Thr}$ from induced cells by derivatisation of Thr-tRNA with naphthoxyacetyl-*N*-hydroxysuccinimide [16] and benzoylated DEAE-cellulose chromatography. The $tRNA^{Thr}$ obtained was 60% pure. After discharging, the $tRNA^{Thr}$ isoacceptors were separated on RPC-5 [17] as shown in fig. 1. The material under each peak was pooled and concentrated and designated $tRNA_1^{Thr}$, $tRNA_2^{Thr}$, etc.

3.2. *Detection of the enzymic activity*

The enzymic activity responsible for the incorporation of threonine into t^6A was first detected in the 100 000 *g* supernatant of alumina ground *E. coli* cells extracted with buffer A. Further purification was on DEAE-cellulose, where the desired enzyme activity was eluted from the column with 0.5 M sodium chloride in buffer A, after it has been thoroughly washed with 0.2 M sodium chloride in biffer A. The detection of t^6A formation was carried out as described in Materials and Methods. While we always found incorporation of [^{3}H] threonine into t^6Ap, the absolute amount was variable. In our best experiments 1 A_{260} unit containing 1 nmole of $tRNA^{Thr}$ (or 0.2 nmoles of undermodified $tRNA^{Thr}$) was found to incorporate 13 200 (3 pmoles) of [^{3}H] threonine into the spot scraped from the plate. With the 25% recovery of material during the extraction procedure this represents an efficiency of modification of 5% of the undermodified material. In order to standardize the results in different experiments, we calculated the ratio of [^{3}H]/[^{32}P] counts recovered as t^6Ap. A representative result, the time course of incorporation of threonine into t^6Ap is shown in fig. 2.

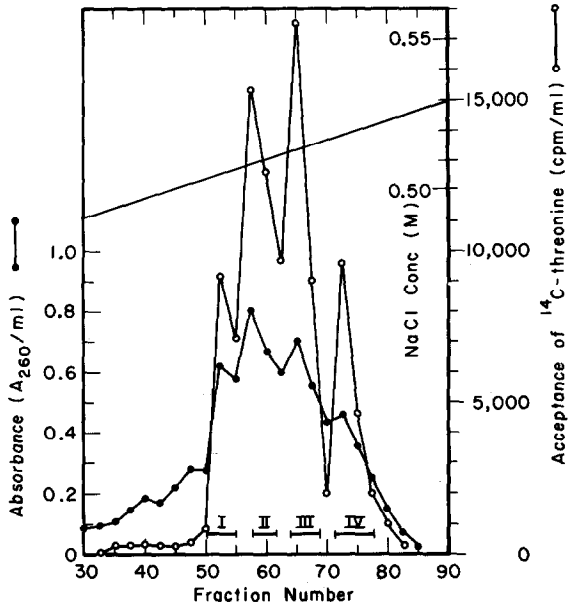

Fig. 1. Separation of $tRNA^{Thr}$ by RPC-5 chromatography. $tRNA^{Thr}$ (40 A_{260} units) partially purified by BD-cellulose chromatography [16] was applied on top of a column (42.5 × 0.4 cm) equilibrated with 0.5 M NaCl–0.01 M sodium acetate (pH 4.5)–0.01 M $MgCl_2$. Elution was carried out with a salt gradient of 0.5 → 0.55 M NaCl in 0.01 M sodium acetate (pH 4.5)–0.01 M $MgCl_2$. Fractions of 1 ml were collected every 10 min. Peaks were pooled as indicated. The threonine acceptor activities (pmoles/A_{260} unit) of the fractions were: I, 500; II, 750; III, 1000; IV, 950.

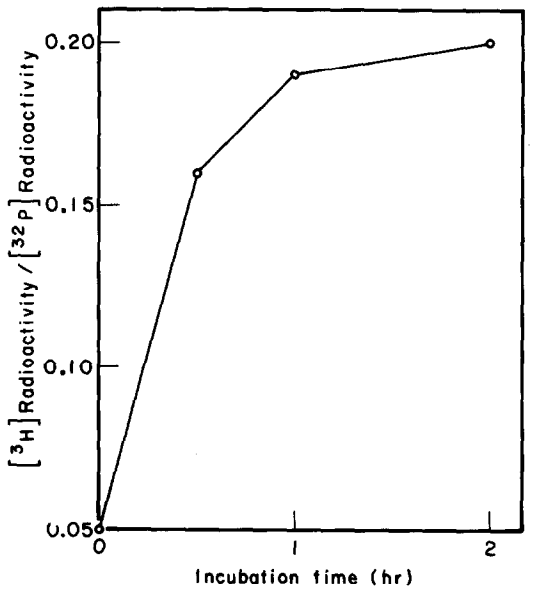

Fig. 2. Biosynthesis of t^6Ap measured as an increase in the ratio $^3H/^{32}P$ radioactivity versus time.

Table 1
Chromatographic and electrophoretic mobilities of authentic and biosynthesized samples of t^6Ap and t^6A *

Compound	Derivation and detection	R_F		Electrophoresis at pH 7 (cm from origin)
		Solvent I	Solvent II	
t^6Ap	Authentic sample	0.39	0.42	–
	^{32}P labelled	0.39	0.42	–
t^6A	Authentic sample	0.75	0.41	10
	^{14}C labelled	0.72	0.43	4.5
Threonine	Authentic sample	0.55	0.40	3
[^{14}C]labelled t^6A alkali treated	^{14}C radioactivity	–	–	3

* Unlabelled nucleotides and nucleosides were detected by their absorbance at 260 nm. Labelled compounds were detected by liquid scintillation counting of paper strips. Threonine was detected by the ninhydrin colorimetric assay.

3.3. *Characterisation of t^6A and t^6Ap*

The product of the reaction, t^6Ap, was analysed at the nucleotide and nucleotide level by cochromatography with standards of t^6Ap and t^6A, respectively. tRNA from several incubations of 1 A_{260} of purified $tRNA^{Thr}$ was digested with T2 ribonuclease and the mononucleotides obtained were separated by two-dimensional thin-layer chromatography. The spots corresponding to t^6Ap were eluted, rechromatographed together in solvent II and then cochromatographed with unlabelled authentic t^6Ap. The coincidence of the UV-absorbing material and the ^{32}P containing spot detected by autoradiography indicated that our product was t^6Ap. For characterization at the nucleoside level the modification reaction was performed with [^{14}C] threonine only. [^{14}C]t^6Ap was isolated from a large scale reaction by two-dimensional paper chromatography. This nucleotide was enzymatically dephosphorylated. When the resulting [^{14}C]t^6A was cochromatographed in two systems with an authentic marker slight differences in the chromatographic mobilities were detected (table 1). However, when the radioactive nucleoside was hydrolyzed with alkali [8] and subjected to paper electrophoresis, the radioactivity was recovered as threonine. Possibly, chromatography of t^6Ap in the highly acidic solvent B converted it to an ester, which was subsequently cleaved again; alternatively, our original compound was the amide derivative of t^6A, which was recently discovered in *E. coli* tRNA (S. Nishimura, personal communication). Furthermore, slight variations in the R_f values of t^6A have been observed previously [9].

3.4. *tRNA is substrate for the enzyme*

A survey of possible RNA substrates for the enzyme showed that the enzyme requires tRNA as a substrate. Derivatives of adenine like Ap, pA and A could not be modified. Furthermore, no incorporation of threonine was observed with bacteriophage f2 RNA, ribosomal RNA, *E. coli* $tRNA_f^{Met}$ or *Mycoplasma sp.* Kid $tRNA^{Ile}$. Only the undermodified *E. coli* tRNA and to a small extent unfractionated *Mycoplasma* tRNA were substrates. Since undermodified *E. coli* $tRNA^{Thr}$ was separated by RPC-5 chromatography into 4 peaks, each of the four tRNA pools (see fig. 1) was tested for its ability to be modified to contain t^6A. The results are shown in table 2. As expected, $tRNA_4^{Thr}$ is the most undermodified isoacceptor species. The incorporation of threonine into the other tRNAs indicates that all $tRNA^{Thr}$ species are partially undermodified and that the column may not fully resolve undermodified and modified tRNA.

3.5. *Characterization of the enzymatic reaction*

The pH optimum for the enzyme was found to be 7.6. There was a strong requirement for ATP, the other ribonucleoside triphosphates were not active.

Table 2
Incorporation of [^{14}C]threonine into t^{6}Ap in four tRNA^{Thr} fractions from RPC-5 chromatography (fig. 1)*

Peak	1	2	3	4
Total tRNA concentration per assay (A_{260} units)	0.10	0.13	0.12	0.08
[^{14}C]threonine incorporated (cpm/assay)**	14	54	134	144
[^{14}C]threonine incorporated (cpm/A_{260} unit) of tRNA^{Thr})***	1510	3530	7200	12430

* For details see Materials and methods. The total RNA concentration in all assays (160 μl) was 4 A_{260} units with carrier rRNA added.
** From thin-layer chromatogram.
*** Data are calculated on the basis of 25% recovery.

Since the incorporation observed is moderate (probably because of the multi-enzyme reaction and the uncertain nature of the immediate tRNA precursor, see Discussion) other compounds which could possibly donate a C_1 fragment were included in the reaction. Formyltetrahydrofolate, S-adenosylmethionine and biotin did not lead to increased incorporation.

4. Discussion

The biosynthesis of t^{6}A is probably accomplished by a complex mechanism. Adenosine or inosine in tRNA could be the starting nucleoside which then is condensed with the reaction product of threonine and an active C_1 donor, or threonine could react with an intermediately modified adenosine. The intermediate products and the origin of the C_1 fragment are not known. The sequence of reactions is probably catalyzed by several enzymes.

We have detected in extracts of *E. coli* the enzymic activity responsible for incorporating threonine into t^{6}A in tRNA. Although *Mycoplasma* tRNA is readily available and devoid of t^{6}A [18] we chose the homologous tRNA as substrate. This was done because *Mycoplasma* tRNA, while lacking t^{6}A, may have the relevant adenosine modified. In fact unfractionated *Mycoplasma* tRNA could be modified to contain t^{6}A under our conditions to about 1/10 the extent of purified tRNA^{Thr} from *E. coli*. As with other tRNA modifying enzymes [7] macromolecular tRNA seems to be the substrate. It is pertinent to note that ATP was found to be required in the enzymic reaction and that the addition of some high speed supernatant fraction to the DEAE-cellulose enzyme increased threonine incorporation. This appears plausible in view of the complex reaction scheme of t^{6}A formation. We do not know the nature of the immediate tRNA precursor of the threonine incorporating enzyme. Thus the low and variable yields in the modification reaction, as well as the need for several enzyme fractions, may mean that the homologous substrate from the lysogen has to be processed by several enzymes before threonine can be incorporated. This uncertainty will make any further enzyme purification difficult.

Concurrent with the investigation of the biosynthesis of t^{6}A we examined the in vitro biosynthesis of its N^6-methyl derivative. The location of mt^{6}Ap on a two-dimensional thin layer chromatogram has been determined by Harada et al. [19]. On inclusion of S-adenosylmethionine in our incubation mixture we were able to demonstrate incorporation of ^{3}H radioactivity from [^{3}H]threonine into the spot corresponding to mt^{6}Ap. The increase in radioactivity was time-dependent and correlated with a concomitant decrease in radioactivity in t^{6}Ap. However, we have not yet isolated and unequivocally identified this material.

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