BIOSYNTHESIS AND SPECIFIC LABELING OF N-(PURIN-6-YLCARBAMOYL)THREONINE OF ESCHERICHIA COLI TRANSFER RNA

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Summary: A relaxed control multiple amino acid auxotroph of Escherichia coli (CP79) incorporated substantial amounts of threonine into transfer RNA during amino acid starvation. The radioactivity in tRNA was localized exclusively in N-(purin-6-ylcarbamoyl)threonine (PCT) and in a modified form of this compound. A mutant with a presumed defect in carbamyl-phosphate synthesis incorporated threonine into PCT, suggesting that carbamyl-phosphate is not the donor of the carbonyl group of the urea moiety of PCT.

Chheda et al. (1) found small amounts of a base containing threonine in acid hydrolysates of tRNA. The structure of the base was determined by Schweizer et al. (2) to be N-(purin-6-ylcarbamoyl)threonine (PCT) (Fig. 1). Takemura et al. (3) discovered PCT adjacent to the anticodon in $tRNA^{ile}$ of T. utilis. PCT was subsequently found in $tRNA^{met}$, $tRNA_3^{ser}$ and $tRNA^{lys}$ from E. coli by Ishikura et al. (4), who suggested that its occurrence might be restricted to those tRNA's whose codons begin with adenine.

The studies presented here show that threonine is the precursor of the threonine moiety of PCT. We describe a procedure for efficiently incorporating a radioactive label into PCT in tRNA and also present data relative to the precursor of the carbonyl group of the urea moiety of PCT.

MATERIALS AND METHODS: [^{14}C]-threonine (208 mc/mmole) and [^{3}H]-threonine (0.8 - 1.5 C/mmole) were purchased from Schwarz/Mann, Orangeburg, New York. Authentic PCT was a gift from Drs. Jerome McDonald and Nelson J. Leonard of the Department of Chemistry, University of Illinois, Urbana, Illinois. RNase T_1

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(Sankyo) and RNase CB were obtained from Calbiochem, San Diego, Calif. Pancreatic RNase and E. coli alkaline phosphatase were obtained from Worthington

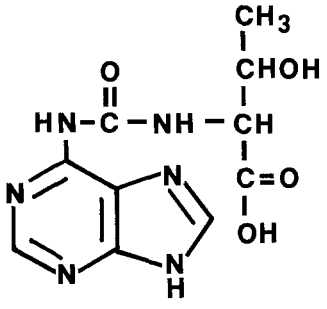

Fig. 1. N-(purin-6-ylcarbamoyl)threonine (PCT).

Biochemical Corp., Freehold, New Jersey. Liquifluor was purchased from New England Nuclear Corp., Boston, Mass. E. coli CP79 (thr^-, arg^-, his^-, leu^-, thi^-, RC^{rel}) and E. coli 15 TAU (thy^-, arg^-, ura^-) were a gift of Dr. Robert Lazzarini. E. coli 15 T^- (thy^-) was a gift of Dr. Theodore R. Breitman.

Radioactivity in tRNA was measured either by collecting TCA-precipitated samples on glass fiber filters and counting the dried filters in Liquifluor-toluene or by counting aliquots directly in Liquifluor-toluene-Triton X-100 (1:12:6). Bacteria were grown on a New Brunswick gyrating shaker at 37° in minimal salts medium (5) with supplements as indicated in the text.

Enzymatic hydrolysis of tRNA was carried out as follows: Samples of radioactive tRNA were dissolved in H_2O and pH was adjusted to 5. After addition of 40 µg RNase CB, the sample was incubated for 6-8 hr at 37°. The pH was then adjusted to 7.5 with NH_4HCO_3 and pancreatic RNase (10 µg) and T_1RNase (10 µg) were added. Incubation at 37° was continued overnight. The sample was lyophilized and a portion analyzed by paper chromatography. Further digestion to nucleosides was accomplished by the addition of alkaline phosphatase (4 µg) and incubation for 8 hr at 37°.

RESULTS: The structure of PCT suggested that threonine might be the precursor of the threonine moiety of PCT. We tested this hypothesis by depriving an amino acid auxotroph with relaxed control of RNA synthesis for its required amino acids. E. coli CP79 (6) is a relaxed-control multi-amino acid auxotroph

(thr^-, arg^-, his^-, leu^-). It was grown in minimal salts medium (500 ml) with the appropriate amino acids (50 µg/ml) and vitamin (2 µg/ml) and 0.3% glucose as the carbon source. When the culture reached a density of 0.5 OD_{650}, the cells were centrifuged and washed two times with 80 ml portions of salts medium without supplements. The washed cells were resuspended in 500 ml of salts medium (amino acid deficient) containing thiamine (2 µg/ml), uracil, adenosine and guanosine (all at 50 µg/ml), and glucose (0.3%). The culture was shaken at 37° for 20 min; at this point 6.5 µmoles of threonine containing either 4 mc of ^3H or 400 µc of ^{14}C were added. Incubation at 37° was continued for 4 to 6 hr, then the cultures were extracted with an equal volume of 88% phenol. The aqueous layer after re-extraction with phenol, was applied to a column of DEAE cellulose (5 ml bed volume); the column was washed with 50 mM potassium cacodylate, pH 7, containing 0.3 N sodium chloride. When the radioactivity in the effluent dropped to a constant low level (after about 25 ml), the tRNA was eluted from the column with 50 mM potassium cacodylate, pH 7, containing 1 N sodium chloride (11 ml). 48% of the radioactivity in the phenol-extracted aqueous fraction was recovered in the 1 N NaCl fraction. tRNA was precipitated with 2.5 vol of cold ethanol. The precipitate was dissolved in 5 ml of 0.1 M Tris-chloride, pH 8, containing 5 mM 2-mercaptoethanol. About 400 A_{260} units of RNA were recovered at this stage. After a 3-hr incubation at room temperature, all of the threonine attached to tRNA in aminoacyl linkage had been hydrolyzed. The tRNA was precipitated with ethanol and dissolved in 1 ml of 50 mM potassium cacodylate, pH 7, containing 0.1 N sodium chloride. 31% of the radioactivity in the phenol-extracted aqueous fraction was recovered. At this stage essentially all of the radioactivity was localized in tRNA as evidenced by the data shown in Fig. 2. Fractionation of the radioactive tRNA on Sephadex G-100 showed that 82% of the radioactivity chromatographed in the tRNA fraction (peak at 85 ml). About 13% of the radioactivity eluted at about 65 ml. Heating this fraction in 0.1 mM EDTA at 60° for 5 min resulted in a change in the elution position to that characteristic of tRNA. This suggests that the minor fraction consisted of dimers of tRNA (7). The 5% of radioactivity eluting at the void

volume is probably residual protein or larger aggregates of tRNA in the preparation.

It has previously been shown (2) that PCT is released from tRNA as the purine base by acid hydrolysis. The nature of the radioactive material in the labeled tRNA was characterized as shown in Fig. 3. After 5 min of acid hydrolysis about 40% of the radioactivity co-chromatographed with authentic PCT,

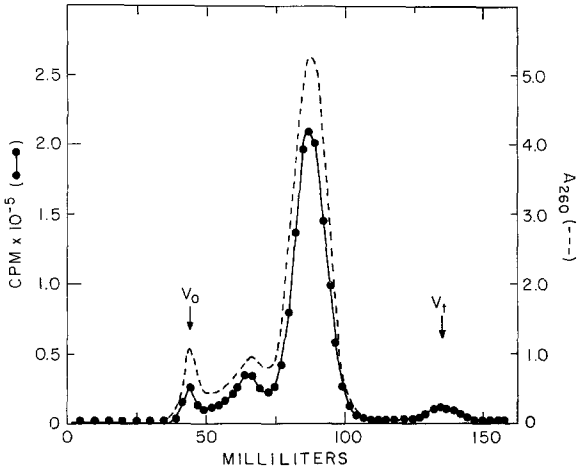

Fig. 2. Sephadex G-100 chromatography of tRNA from threonine-labeled E. coli. Transfer RNA from threonine-labeled cells was prepared as described in the text. 208 A_{260} units containing 3.25 x 10^6 cpm of [^{3}H]-tRNA (deacylated DEAE-cellulose fraction) was applied to a column (1.5 x 90 cm) of Sephadex G-100 equilibrated with 0.05 M potassium cacodylate, pH 7.0, containing 0.1 M NaCl. Chromatography at room temperature was continued with the same buffer. Alternate fractions (1.25 ml) were assayed for absorbance at 260 nm. Radioactivity was determined by counting aliquots in Triton-toluene-Liquifluor and is expressed as total cpm per fraction. Calibration of the column with aminoacyl-tRNA showed that tRNA elutes at about 85 ml. V_o indicates the void volume of the column; V_t indicates the total volume.

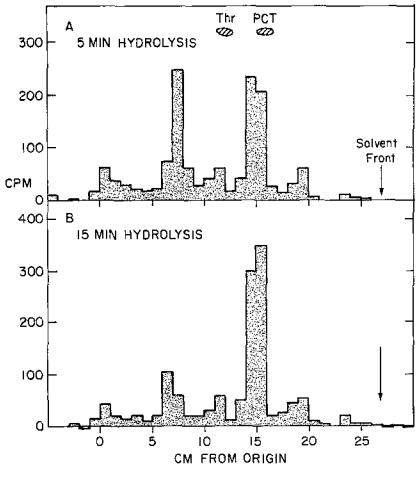

Fig. 3.

Fig. 3. Paper chromatography of acid hydrolysate of [^{14}C]-tRNA after different times of hydrolysis. About 16,000 cpm of [^{3}H]-tRNA (Sephadex G-100 fraction) in 0.5 ml of 0.5 N HCl was heated in a boiling water bath for either 5 or 15 min. After evaporation of the HCl, the samples were chromatographed (ascending) on Whatman 3 MM paper. The dried paper was cut into 1 cm strips and counted in 10 ml of Liquifluor-toluene (1:24, v/v) in a Packard scintillation counter.

while approximately 30% of the radioactivity was accounted for by another compound with a slower Rf. Continued acid hydrolysis led to disappearance of the slower-moving radioactive compound concomitant with the appearance of the PCT-like compound. This suggests that the majority of PCT may exist naturally in a modified form. Further identification of the radioactive compound as PCT is

TABLE 1

Identification of labeled PCT in tRNA from threonine-labeled E. coli

	Rf Values				
	A	B	C	D	E
PCTR+	.04	.60	---	---	---
Labeled tRNA					
1) nuclease treated	.49	.49	---	---	---
2) nuclease and phosphatase treated	.02	.70	---	---	---
3) acid hydrolyzed	---	.59	.63	.48	.25
PCT standard	.31	.62	.59	.45	.28
Threonine standard	.35	.45	.47	.34	.50

See the text for details. Paper chromatography was carried out as in Fig. 3.

+ Values according to Ishikura et al. (4).

A n-Butanol:H_2O:NH_4OH (86:14:5).

B Isobutyric acid:NH_4OH:H_2O (66:1:33).

C 2-Propanol:1% aqueous $(NH_4)_2SO_4$ (2:1)

D 2-Propanol:H_2O:NH_4OH (7:2:1).

E 2-Propanol:HCl:H_2O (650:176:144).

shown in Table 1. Samples of radioactive tRNA were digested with a mixture of nucleases. Chromatography of the digest in systems A and B showed the principal radioactive compound eluting at Rf 0.49 in both systems. Treatment of this presumed nucleotide of PCT with alkaline phosphatase resulted in a new compound which migrated with Rf's 0.02 (system A) and 0.70 (system B). The lack of complete agreement of these Rf's with those determined by Ishikura et al. (4) for the authentic ribonucleoside of PCT (PCTR) might be due to differences in

chromatographic technique or might suggest that our ribonucleoside is a further modified form of PCTR as evidenced by the data of Fig. 3. A second component of the nuclease digest was resistant to treatment with phosphatase and pronase. This is consistent with it being the 2',3'-cyclic nucleotide of PCT as reported by Ishikura et al. (4). Examination of acid-treated samples (15 min hydrolysis as in Fig. 3) in systems B, C, D and E showed that in all cases the major radioactive product migrated with authentic PCT (Table 1).

The demonstration that threonine is a precursor of the threonyl group of PCT still leaves open the question of the origin of the carbonyl group of the urea moiety. An experiment was performed to test the possibility that carbamyl phosphate is the donor of the carbonyl group. E. coli TAU requires arginine and uracil. Since this double auxotrophy is the result of a single-step mutation, it has led to the speculation that the mutant has a defect in the biosynthesis of carbamyl phosphate (8,9). If carbamyl phosphate were required for the biosynthesis of PCT in tRNA, tRNA from this mutant should not contain the PCT modification. The results of an experiment to test this hypothesis are described in Table 2. When E. coli TAU was grown in a medium containing

TABLE 2

Incorporation of radioactive threonine into tRNA in different E. coli strains

Strain	tRNA specific activity (cpm/A_{260} unit)
15 T^-	2400
TAU	3280

Overnight cultures of 15 T^- or TAU were diluted into 250 ml of fresh salts medium (5) supplemented with thymine, uracil, adenosine and guanosine (all at 50 μg/ml) and arginine at 100 g/ml. The carbon source was glucose (0.3%). The initial OD_{650} was 0.25. 200 μc of [^{14}C]-threonine (210 mc/mmole) was added to each culture, followed by incubation with shaking for 3 hr (final OD_{650} = 1.5). The cells were harvested and washed by centrifugation. Transfer RNA was prepared as described in the text, except that the tRNA was deacylated before DEAE-cellulose chromatography. tRNA was eluted from DEAE-cellulose by a gradient (0.25 M NaCl, 50 mM potassium cacodylate, pH 7 -- 1.0 M NaCl in the same buffer). The specific activity of the tRNA was determined in the DEAE-cellulose fractions by counting samples after TCA-precipitation.

radioactive threonine, the specific activity of the tRNA recovered was at least as great as that recovered from a strain with no defect in carbamyl phosphate synthesis. Chromatography of the acid-hydrolyzed tRNA in system B established that the radioactivity in the tRNA isolated from both strains was indeed PCT.

DISCUSSION: The technique of utilizing amino acid starvation of a relaxed-control bacterium to label PCT in tRNA may be useful for a variety of biochemical problems. In studies to be published elsewhere we have taken advantage of this specific labeling to demonstrate that those tRNA's containing radioactivity are capable of accepting a limited number of amino acids. In this way it was possible to show that PCT is found in all of the tRNA's whose codons begin with A.

Transfer RNA labeled by the procedure described here contains at least 50% of the PCT in a form that is probably further modified. Ishikura et al. (3) have observed other nucleosides which were similar in spectrum to PCT but different in chromatographic properties. It is not certain if they are modified forms of PCT or analogs of PCT containing other amino acids [such as N-(purin-6-ylcarbamoyl) glucine (11)] which would exhibit similar spectral properties. Another minor base, 5-carboxymethyluracil, has been shown to exist in tRNA as a methyl ester (10).

The biosynthetic pathway for PCT and its modified form in E. coli tRNA is not yet clear. The results presented here indicate that threonine is the precursor of the threonyl-moiety of PCT. The anticipation that carbamyl phosphate might be the precursor of the carbonyl group of the urea moiety of PCT was not supported by the data presented in Table 2. The reported leakiness of the uracil and arginine requirement of E. coli TAU (8) introduces the possibility that the organism is capable of synthesizing decreased levels of carbamyl phosphate sufficient to satisfy the requirements for biosynthesis of PCT. Alternatively, since biotin is involved in many carboxylation reactions, we can speculate that a biotin-dependent reaction may be involved in the biosynthesis of the carbonyl group. Experiments utilizing PCT-deficient tRNA in vitro should be useful in further elucidating the biosynthetic pathway of this unusual nucleotide.

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