SEPARATION AND CHARACTERIZATION OF AN INTRAMOLECULAR CROSS-LINKED FORM OF $tRNA^{Met}_{f}$ FROM *E. COLI*

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1. Introduction

Chromatographic heterogeneity of an amino acid specific tRNA generally reflects the multiplicity of isoacceptors naturally occurring in the cell. However, it may also reflect the presence of tRNA molecules that have been modified during or after the extraction procedure. Shugart et al. [1] reported the separation on reverse phase chromatography columns of two distinct forms of *E. coli* $tRNA^{Met}_{f}$. These authors showed that the modified form – called $tRNA^{Met}_{f2}$ – contained a much lower amount of 4-thiouridine (4TU) than the normal form of $tRNA^{Met}_{f}$, $tRNA^{Met}_{f1}$.

We have observed a similar behaviour of *E. coli* $tRNA^{Met}_{f}$ on benzoylated DEAE columns. The modified form was characterized: the 4TU in the 8th position in the tRNA sequence is actually cross-linked with a cytosine residue in the 13th position.

2. Materials and methods

Crude *E. coli* tRNA was prepared according to Zubay [2] and $tRNA^{Met}_{f}$ purified as described by Seno et al. [3]. The enzymatic fractions used for acylation were obtained according to the method of Muench and Berg (DEAE-cellulose step) [4]. The procedure used for 335 nm UV irradiation of tRNA was as previously described [5]. It resulted in the cross-linking of a 4-thiouridine with a cytosine residue (TU–C) in a number of *E. coli* tRNAs including $tRNA^{Met}_{f}$ [5]. The irradiated $tRNA^{Met}_{f}$ fractions (f. irr.) used in this work were cross-linked to completion (irradiation was performed for 2 hr in the conditions of [5]. The TU–C photodimer was detected using the fluorescence of its sodium borohydride reduction product [6]. Fractionation of the RNase T_1 digest of $tRNA^{Met}_{f}$ was done according to Schulman [7].

3. Results

Following the procedure of Seno et al. [3], crude *E. coli* tRNA was first chromatographed on a DEAE-Sephadex A50 column yielding a methionine tRNA enriched fraction which was further purified on BD-cellulose columns. In the elution profile of these columns, we have frequently observed the presence of an extra peak called $tRNA^{Met}_{f2}$, just following the normal $tRNA^{Met}_{f}$ position ($tRNA^{Met}_{f1}$) and preceeding $tRNA^{Met}_{m}$ (fig. 1). When re-run on a BD column, the f1 fraction eluted as a single peak. In different preparations, the relative amount of f2 with respect to f1 plus f2 varied from 25 to 65%. The two subspecies were further characterized. Acceptor activities with the homologous synthetase were, respectively, 1.350 and 1.200 pmoles of methionine per A_{260} unit. The rabbit liver methionyl-tRNA synthetase which did not acylate the *E. coli* $tRNA^{Met}_{m}$ [8] partially recognized the f1 and f2 fractions.

Very similar results have been reported by Novelli's group who concluded that $tRNA^{Met}_{f2}$ arises from $tRNA^{Met}_{f1}$ as a result of a chemical modification of

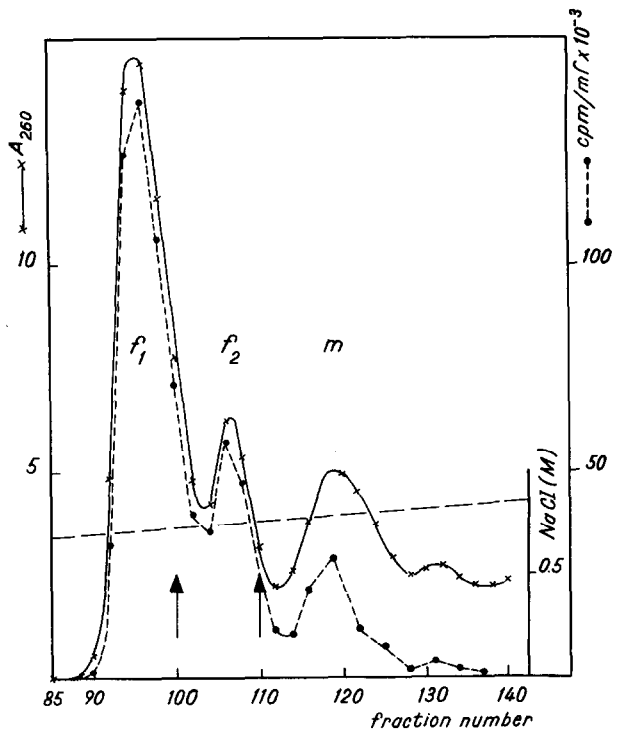

Fig. 1. BD-cellulose chromatography of *E. coli* $tRNA^{Met}$. $tRNA^{Met}$ (845 A250 units in 5 ml of 0.02 M sodium acetate buffer pH 6, 0.4 M NaCl) was absorbed onto a BD-cellulose column (1 × 100 cm) equilibrated with the same buffer at 4°. A linear gradient of NaCl (0.4 – 1.4 M) was then applied (total vol 800 ml). The rate of elution was about 6 ml/hr. Absorbance at 260 nm (×——×——×) and methionine acceptor activity (•——•——•) were measured on each fraction. The fraction f2 used throughout this work was taken as indicated by the two vertical arrows (fractions 100 to 110).

Table 1
TU–C content of the $tRNA^{Met}_{f1}$ and $tRNA^{Met}_{f2}$ forms.

	$tRNA^{Met}_{f1}$	$tRNA^{Met}_{f2}$
before 335 nm irradiation	44	160
after 335 nm irradiation	280	240

The TU–C photoproduct present in the tRNA fractions was measured using the fluorescence (reported here in arbitrary units) of its $NaBH_4$ reduction product. Measurements were performed on 1 A260 unit of tRNA at 25° in 0.15 M Na cacodylate, 0.01 M $MgCl_2$ pH 7 buffer.

the 4-thiouridine [1]. It has been shown that irradiation at 335 nm of a number of *E. coli* tRNAs including $tRNA^{Met}_f$ induced the formation of a 4-thiouridine-cytosine (TU–C) photodimer*. Using the fluorescence method previously described [6], we detected the TU–C content was much higher in the f2 (65%) than in the f1 form (15%). In order to identify positively f2 fraction with the 335 nm irradiation product of $tRNA^{Met}_f$–f. irr.–we compared the chromatographic behaviour of these two fractions and of their T_1 digestion products. A mixture of f1 and f.irr. was chromatographed on BD-cellulose. Fig. 2 shows that f.irr. eluted just after f1, i.e. in a position very similar to that of f2 (compare figs. 1 and 2). As a control, f.irr. run alone on a BD-column showed a single elution peak as followed by UV absorption, fluorescence and acceptor activity (fig. 3).

Additional evidence that f2 was really identical to f.irr. was provided by analysis of the T1 digest of each species on DEAE-cellulose columns (fig. 4). Starting from $tRNA^{Met}_{f1}$, we observed a single 335 nm absorption peak that we assigned to the dinucleotide 4 TUG. The f2 form gave two 335 nm absorbing peaks, the first one corresponding to 4 TUG, the second eluting simultaneously with the hexanucleotide CCCCCG. Starting from f.irr., only this second peak was obtained. This 335 nm absorbing oligonucleotide was separated from the contaminating CCCCCG oligonucleotide on a DEAE-cellulose–urea column at pH 3 and was shown to contain the TU–C cross-link. The presence of a 335 nm absorbing peak, eluted in the same region of the chromatogram, has been reported by Schulman [7] in digest of $tRNA^{Met}_f$ irradiated at 254 nm.

4. Discussion

Our findings support the conclusions of Shugart and al. [1] and, in addition, state on the nature of the 4-thiouridine modification. Our approach was based on the comparison of the $tRNA^{Met}_{f2}$ form that appears during purification with $tRNA^{Met}_f$ irradiated at 335 nm. Both the f2 and f.irr. fractions had the same chromatographic

* The chemical nature of the TU–C photodimer is actually known: it is the riboside of 5-(4'-pyrimidin-2'-one) cytosine [9,10].

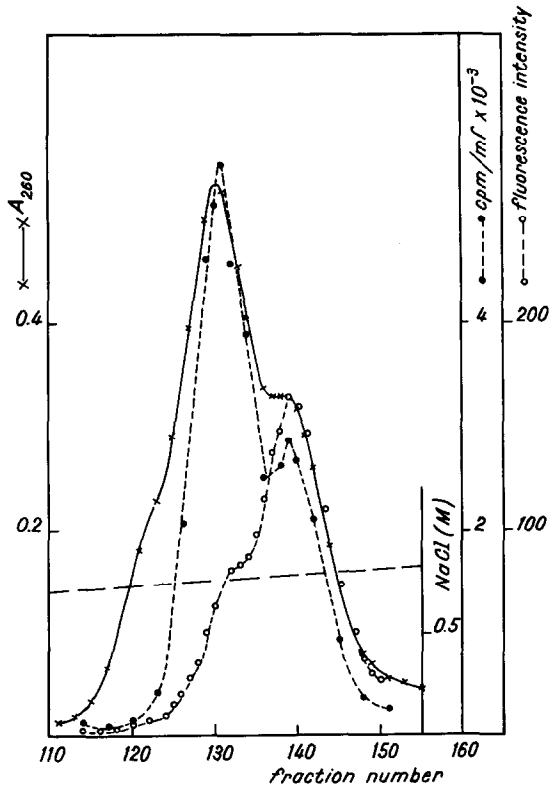

Fig. 2. Co-chromatography of $tRNA^{Met}_{f1}$ and $tRNA^{Met}_{f.irr.}$ in a ratio of 2 to 1 on a BD-cellulose column. The conditions were those of fig. 1. Elution of tRNAs was followed by 260 nm absorbance (×——×——×) and methionine acceptance (●——●——●). In addition aliquots of each fraction were reduced with $NaBH_4$ and their fluorescence (λ ex. 390 nm – λ em. 450 nm) measured (○——○——○). This is reported here in arbitrary units.

behaviour on BD column. In addition, their T_1 digests contain a large 335 nm absorbing oligonucleotide. This oligonucleotide, obtained from irradiated $tRNA^{Met}_{f}$ has not been definitively identified. However, from its chromatographic behaviour at pH 7.5 and pH 3 on DEAE-cellulose–urea columns and from what we knew about the TU–C cross-link formation in different *E. coli* specific tRNAs [5], we can propose that this oligonucleotide is most certainly $\overline{TUG\ CAG}$. This finding was also in agreement with previous observations of Dube et al. [11] and Schulman [7].

The difference observed between f2 and f.irr. can be understood since the f2 form used in this work was obtained by pooling the fractions eluted from the BD column as shown in fig. 1. This is in accord with: i) the nonquantitative content of TU–C in the f2 form as well as the presence in its RNase T_1 digest of some 4 TUG dinucleotide. ii) The presence of some TU–C in the f1 form. It should be pointed out that contamination of f1 by f2 is not only due to incomplete chromatographic separation. The presence of a shoulder in the fluorescence curve of fig. 2 indicates that a small amount of cross-linked molecules are eluted together with $tRNA^{Met}_{f1}$. Control experiments show that monitoring the elution of the BD column in the LKB densitometer increases by 5% the TU–C content of the f1 form. However, the presence of low quantities of tRNA dimers cannot be totally excluded. We can thus conclude that f2 differs from f1 only by its high TU–C content.

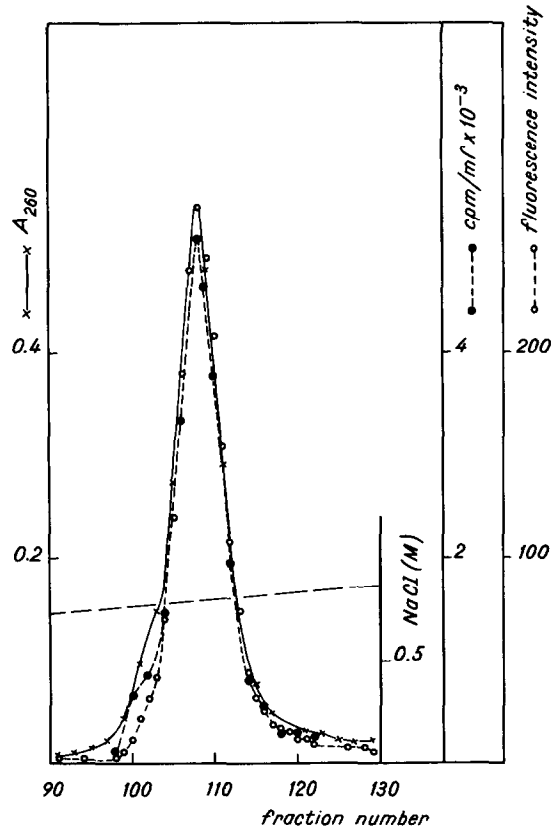

Fig. 3. Chromatography on a BD-cellulose column of irradiated $tRNA^{Met}_{f}$. The conditions were those of figs. 1 and 2. (×——×——×) absorbance at 260 nm; (●——●——●) methionine acceptance; (○——○——○) fluorescence (390 – 450 nm) in arbitrary units.

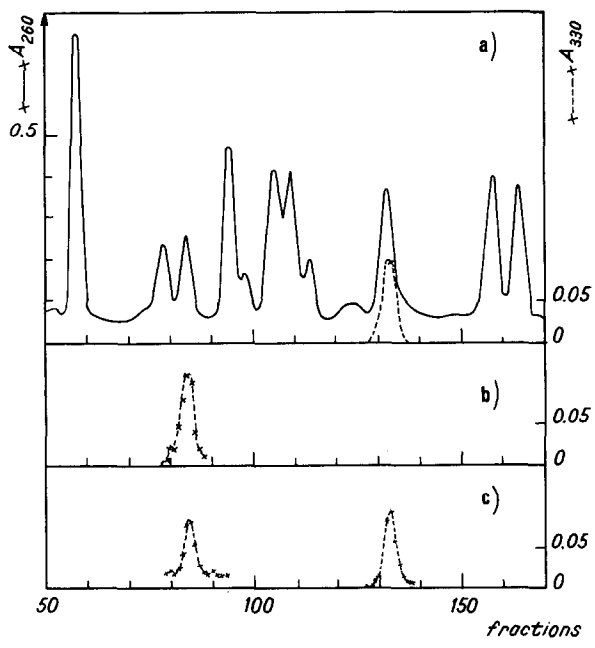

Fig. 4. Fractionation on a DEAE-cellulose–urea column (0.6 × 105 cm) of the RNase T_1 digests of different *E. coli* $tRNA^{Met}$ fractions. Elution was performed with a NaCl gradient from 0 to 0.6 M (in 0.02 M Tris pH 7.5 in the presence of 7 M urea; total vol 800 ml). Elution was followed at 260 nm and showed a very similar profile for the three samples. Localization of the 335 nm absorbing peaks is represented in a) for irradiated $tRNA^{Met}_f$; in b) for $tRNA^{Met}_{f1}$ and in c) for $tRNA^{Met}_{f2}$.

The next question is: how does this conversion occur? Although the TU–C content of total *E. coli* tRNA is low (in different commercial samples it never exceeds 5% of its maximum value) it is generally between 15 and 30% in purified specific tRNAs (unpublished results). Consequently, the cross-link is formed during tRNA purification. It is essentially mediated by visible light since a 100% conversion is observed in a total *E. coli* tRNA sample abandoned 2 days in the presence of sunlight. Since a number of *E. coli* tRNAs can be cross-linked [5], presumably the type of heterogeneity we report here for $tRNA^{Met}_f$ also occurs in other species although $tRNA^{Met}_f$ represents a particularly favorable case for the chromatographic separation of native and cross-linked tRNA molecules.

So far, variable estimations have been made of the yield of conversion of 4 TU into TU–C photoproduct in purified tRNA. For example, in $tRNA^{Val}_1$, this yield was about 85% as measured by the finger-print technic and 95% by optical methods [5]. In the case of $tRNA^{Met}_f$, the chromatographic separation of the intact and irradiated molecule provide an alternative approach. From the elution profile of irradiated $tRNA^{Met}_f$ (fig. 3) it is clear that 95% of the biologically active material is in the cross-linked form, a value close to that estimated by optical technics.

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