CHEMICAL STRUCTURES OF THE TU–C AND TU–C^{red} PRODUCTS DERIVED FROM *E. coli* tRNA*

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1. Introduction

In 1969 we observed that irradiation at 335 nm of *E. coli* $tRNA_1^{Val}$ resulted in a specific and quantitative photoreaction of 4-thiouridine (4TU) [1]. The photoreaction takes place between the 4TU in 8 position and the cytidine in 13 position as shown by Yaniv et al. [2]. Moreover, sodium borohydride reduction of the TU–C photodimer quantitatively yields a fluorescent product TU–C^{red}, easily detected in the tRNA [3]. These reactions occur in a number of *E. coli* tRNA [4–5] and they have proved to be of value for the study of tRNA tertiary structure. They were also used to study the functioning of those molecules in the elongation [5–7] and initiation [8] processes of protein biosynthesis.

Clearly a better understanding of these problems requires the determination of the chemical structure of the TU–C and TU–C^{red} products. We have previously described the isolation of TU–C from 335 nm irradiated *E. coli* $tRNA_1^{Val}$ [4]. Since in the range 300–400 nm the absorption spectrum of TU–C remains very similar to that of 4TU it appeared possible that the cross-link formation does not greatly affect the 4TU moiety (substitution at N_3 for example). In the present work this possibility is ruled out: clearly the sulfur is eliminated during 335 nm irradiation of *E. coli* tRNA labelled with ^{35}S. We have also purified TU–C enzymatically from irradiated total *E. coli* tRNA and examined in more detail some of its optical and chemical properties.

At this stage of our work, Rhoades and Wang reported the isolation of a photoproduct of cytidine, namely the diriboside of 5-(4′-pyrimidin 2′-one) cytosine that absorbs at 330 nm [9]. Simultaneously, Leonard et al. proposed the same structure for a photoproduct obtained by irradiation of a mixture of 4- thiouridine and cytidine [10]; using our data these authors identified their compound with authentic TU–C extracted from $tRNA_1^{Val}$.

We have prepared the diriboside of 5-(4′-pyrimidin 2′-one) cytosine following the procedure of Rhoades and Wang and established its identity with authentic TU–C. In addition we have established the structure of the TU–C^{red} product.

2. Experimental

2.1. *Methods*

Measurements of optical properties of the products under study were carried out with a Cary 15 spectro-

* A preliminary communication describing this work was presented by A.F. at the tRNA meeting held in Strasbourg in December 1971.

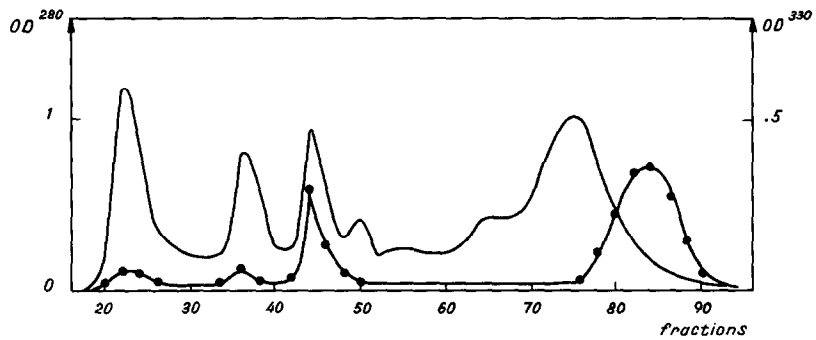

Fig. 1. Purification of TU–C on a P_2 column. (TU–C) containing oligonucleotides were digested extensively by phosphodiesterase in the presence of alkaline phosphatase. After removel of proteins as described in Experimental 40 ml of supernatant were recovered. This supernatant was applied in 10 ml aliquots to a 100–200 mesh P_2 column (60 × 3.5 cm); elution was performed with H_2O and absorbance was followed at 280 nm (——) and 330 nm (–•–•–•–). Fractions 86 to 91 only were considered as pure photodimer.

photometer, a Jouan DC dichrograph and a FICA 55 absolute spectrofluorimeter.

PMR spectra were recorded at 100 MHz on a Varian HA 100 spectrometer (TMS as an internal standard).

2.2. *Preparations*

2.2.1. Isolation of TU–C from *E. coli* tRNA

About 10,000 A_{260} units of tRNA (Schwarz Bioresearch Co.) in 100 ml of 0.15 M sodium cacodylate pH 7 were irradiated at 20° with the Cunow lantern system as previously described [4]. After 10 hr of irradiation about 80% of the 4TU is converted into TU–C as controlled by the fluorescence method [3]. Some tRNA aggregates formed during irradiation and were removed by Sephadex G-100 chromatography. After dialysis against water and concentration by lyophilisation, irradiated tRNA (8,000 A_{260} units) was digested with 10 mg of T_1 RNAase (Sankyo) and 15 mg of pancreatic RNAase in 130 ml of 0.05 M Tris-HCl pH 7.5 and 0.002 M EDTA. After 24 hr at 37° 10 mg of pancreatic RNAase were again added and the solution maintained another day at 37°. The oligonucleotides were then adsorbed onto a DEAE-cellulose column (100 × 5 cm) as described [4]. Elution was performed with a linear gradient (1 l of 7 M urea 0.02 M Tris-HCl pH 7.5 buffer and 1 l of 0.3 M NaCl in the same buffer). Fractions (10 ml) were collected at a rate of 10 ml/hr. A single 330 nm absorbing fraction was eluted at an approximate molarity of 0.2 M NaCl. When the initial input in tRNA was decreased 3-fold, we obtained in addition other 330 nm absorbing fractions that eluted at a lower molarity from the DEAE column. Using the fluorescence test [3] all these 330 nm absorbing fractions were shown to contain the TU–C photodimer. The assembled fractions yielded 100 A_{330} units of oligonucleotides 10-fold enriched in TU–C. The urea was removed by passage over Biogel P_2 columns and the solution concentrated by lyophilisation.

Further digestion of the TU–C containing fraction was achieved in the presence of snake venom phosphodiesterase (Sigma Co.) and bacterial alkaline phosphatase (Worthington) as described [11]. We finally obtained a supernatant which was concentrated by lyophilisation to a volume of 40 ml. Purification of TU–C was achieved on a P_2 column as shown in fig. 1.

2.2.2. Diriboside of 5-(4′-pyrimidin 2′-one) cytosine

This compound was prepared according to the procedure of Rhoades and Wang [9] with a 30% yield.

2.2.3. ^{35}S labelled tRNA

The tRNA was extracted by phenol from the 150,000 *g* supernatant of *E. coli* MRE 600 grown in a minimal medium in the presence of $Na_2{}^{35}SO_4$. The tRNA was stripped, precipitated twice with cold ethanol and finally purified on a Sephadex G-75 column.

2.2.4. Sodium borohydride reduction

Reduction of TU–C was carried out as described [3]. Reduction of the diriboside of 5-(4′-pyrimidin 2′-one) cytosine (38 mg in 15 ml of water) was per-

Table 1
Sulfur content of intact and modified *E. coli* tRNA.

Intact	Irradiated	Reduced
100%	39%	36%

^{35}S labelled *E. coli* tRNA was modified as described in the Experimental section and in the text. Each sample (1 A_{260} unit) was then precipitated by 5% TCA, collected on Whatman 3 MM filters and washed with cold ethanol. The filters were dried and counted in a Beckman liquid scintillator. In table 1 the counts are normalized with respect to intact tRNA (each value is the average of three experiments).

formed by progressively adding 9 mg of $NaBH_4$. After 15 min the pale yellow solution was neutralised with Amberlite IRC 50 (H^+) and lyophilised. The residue was then washed several times with methanol and yielded 36 mg of reduced product.

3. Results

3.1. *Purification of the TU–C photodimer*

The TU–C photoproduct was purified as described in the Experimental section. Chromatography on DEAE-cellulose columns of the T_1 plus pancreatic RNAase digests of 335 nm irradiated tRNA gave with a 60% yield fractions ten times enriched in TU–C. Digestion was then continued to the nucleoside level and TU–C purified on a P_2 column as shown in fig. 1. We finally obtained 50 A_{330} units of TU–C, i.e. a yield of 20% for the overall purification procedure. Of these 50 A_{330} units, 20 were pure TU–C.

3.2. *Sulfur content of TU–C and TU–C^{red}*

No sulfur could be detected in either the TU–C or the TU–C^{red} products, using the Feigl test as described by Ofengand [12]. Furthermore, this was directly confirmed by means of ^{35}S labelled tRNA. The data of table 1 shows that irradiation at 335 nm resulted in considerable elimination of the sulfur atom. Since the 4TU photoreaction is almost quantitative [4] with *E. coli* tRNA and since no other sulfur containing bases are known to absorb at 330 nm, it is clear that the sulfur is removed during the formation of the TU–C cross-link.

As a control of 4TU sulfur elimination a tRNA sample was extensively reduced in the dark with $NaBH_4$.

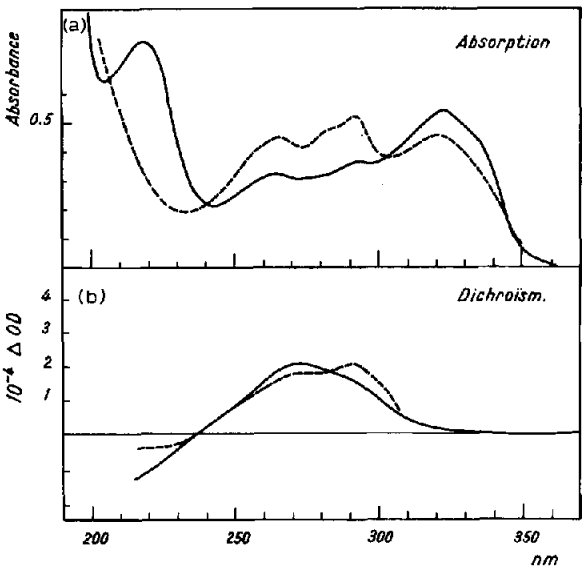

Fig. 2. Optical properties of the (TU–C) photoproduct at pH 2 (----) and pH 7 (——): a) absorption spectra; b) dichroic spectra (0.540 A_{330} unit pH 7 solution); c) fluorescence spectra (arbitrary units).

In these conditions 4TU yielded *N*-ribosyl 2-oxo-hexahydropyrimidine [13]. This 4TU conversion was nearly quantitative as determined by absorbance measurements. From the data of table 1 it appears likely that:

i) sulfur-containing bases other than 4TU are insensitive to extensive reduction conditions;
ii) the sulfur contained in 4TU represents 60 to 65% of the total sulfur found in *E. coli* tRNA;
iii) sulfur elimination from 4TU induced irradiation at 335 nm is nearly quantitative.

3.3. *Optical and chemical properties of TU–C and TU–C^{red}*

The fluorescence and absorption spectra of TU–C isolated as described here are very similar to those previously obtained with TU–C excised from $tRNA_1^{Val}$ [4]. These spectra (fig. 2a and 2c) are very sensitive to protonation: the quantum yield of fluorescence emission increases from 4×10^{-4} E. mole $^{-1}$ at pH 7 to 10^{-3} E. mole^{-1} at pH 2. Yet the TU–C dichroic spectrum is much less affected (fig. 2b).

Based on UV absorption changes we observed that TU–C is fairly stable in aqueous solution in the dark at neutral pH, but decomposed to unknown products

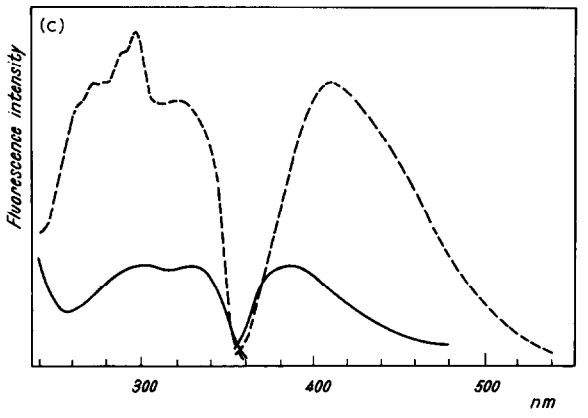

Fig. 2c.

in 0.1 M NaOH with a half-life of about 10 hr at 25°. TU–C could also be decomposed by 335 nm irradiation. The quantum yield for such decomposition is 5×10^{-3} E. mole^{-1} at pH 2 but decreases to 2×10^{-5} E. mole^{-1} at pH 7 (it should be noted that in *E. coli* tRNA the quantum yield of TU–C formation is 5×10^{-3} E. mole^{-1}).

We have not attempted to directly isolate TU–C^{red} from tRNA. This compound was always obtained by reduction of pure TU–C. Absorbance spectra (fig. 3) indicate the presence of two pK's whose values were determined by spectrophotometric titration ($pK_1 = 4$; $pK_2 \sim 11$). Although TU–C^{red} is highly fluorescent in tRNA it is much less so in aqueous solution [3]. No dichroic signal is detected for the longer wavelength transition of both the TU–C^{red} (measurement done with 0.9 A_{380} unit) and TU–C (fig. 2b) products. It should be noticed however that readily detectable dichroic signals are observed in $tRNA_1^{Val}$ when TU–C and TU–C^{red} are in close proximity to adjacent bases (unpublished results).

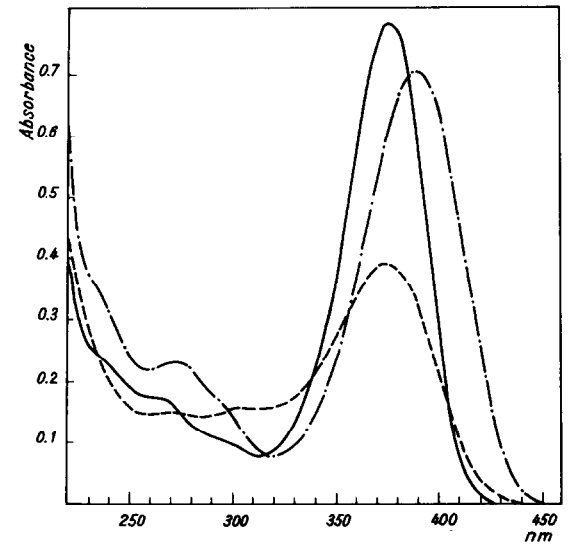

Fig. 3. Absorption spectra of the (TU–C^{red}) product in 0.1 M NaCl at pH 7 (——); pH 2 (----) and pH 12 (–·–·–).

3.4. *Structure of TU–C and TU–C^{red}*

About 50 mg of the diriboside of 5-(4′-pyrimidin 2′-one) cytosine were prepared from cytidine by the procedure of Rhoades and Wang [9]. This cytidine adduct can be reduced by $NaBH_4$*.

The optical and chemical properties of the TU–C and TU–C^{red} products presented above, were then compared respectively to those of the cytidine photodimer and its reduction product; they proved to be identical in every respect. We have thus confirmed the structure proposed by Leonard et al. [10] for the TU–C photoproduct. The TU–C^{red} structure was inferred from the PMR spectrum of the reduction product of the cytidine adduct. In $DMSO_{d_6}$ the spectrum shows no signal at 9.10 ppm corresponding to H_6 in the diriboside of 5-(4′-pyrimidin 2′-one) cytosine. It retains the pair of doublets at 6.16 and 7.33 ppm (J = 7 Hz) assigned respectively to H_8 and H_9 which display an upfield shift. The position of the sugar protons are nearly equivalent in both compounds. The 9.22 and 4.68 ppm signals are due to the amino protons (2 H) and hydroxyl protons (6 H) since they disappear after D_2O addition. Trifluoroacetic acid was added to allow an unambiguous examination of the region between 3 and 4 ppm. Two peaks, one at 3.83 (6 H) and the other at 3.43 ppm (6 H), were observed. The former is assigned to the $H_{2'3'4'}$ sugar protons and the latter to the superposition of the four protons $H_{5'}$ and of the two protons that correspond to the methylene protons in C_6. The determination of the exact configuration of this product (H_8 cis or trans versus C_6) is under investigation.

* By 254 nm irradiation of poly C, Rhoades and Wang claim to obtain the diriboside of 5-(4′-pyrimidin 2′-one) cytosine at both pH 7 and 4 [9]. Using the reduction method we observed such a formation only in conditions where Poly C is in the double-stranded acidic form. A different cytidine adduct formed at pH 7 (Favre, unpublished results).

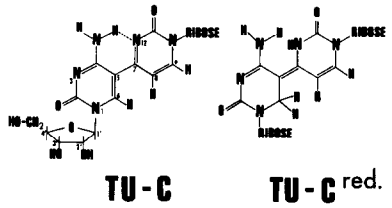

Fig. 4. Structure of TU–C and TU–C^{red}. Numbers in this figure only refer to PMR assignment.

These results suggest that TU–C^{red} is the diriboside of 5-(4′-pyrimidinylidene 2′-one) dihydro 5,6-cytosine (fig. 4). The proposed structure is consistent with the presence of two pK values, the acidic (pH 4) due to the protonation of N_3 of the dihydrocytosine moiety and the basic (pH $\sim$ 11) to deprotonation of the intracyclic nitrogen of the pyrimidinylidene 2′-one moiety. Moreover, the strong conjugation of the π bonds is compatible with the blue shift in absorbance observed during TU–C reduction.

4. Discussion

The isolation of pure TU–C starting from *E. coli* tRNA is reported. This photoproduct results from the cross-linking of TU 8 and C 13 in the tRNA species which have these residues in common [2]. Pure TU–C exhibits identical physico-chemical properties to the diriboside of 5-(4′-pyrimidin 2′-one) cytosine prepared by the procedure of Rhoades and Wang [9]. In addition, elimination of sulfur during 335 nm irradiation of tRNA has been verified. These results confirm the structure proposed by Leonard et al. [10].

It is known that the TU–C link remains stable during $NaBH_4$ reduction [3]. This is in accordance with the structure of TU–C^{red} inferred from PMR spectra and spectrophotometric titration: TU–C^{red} is the diriboside of 5-(4′-pyrimidinylidene 2′-one) dihydro 5,6-cytosine (fig. 4).

One can ask whether the successive introduction of the TU–C and TU–C^{red} products alters the conformation of tRNA in solution. Evidence that the introduction of the TU–C cross-link induces conformational changes elsewhere in the $tRNA_1^{Val}$ molecule has been provided [4]. This finding has recently been confirmed for several *E. coli* tRNA species by a more sensitive approach [14]. Yet we fail to detect any further change upon subsequent reduction. This is quite understandable on the basis of the structures proposed for the TU–C and TU–C^{red} products since the relative positions of the glycosidic linkage may remain unchanged during reduction. In turn, this indicates that these positions have changed during the cross-link formation.

Bergstrom and Leonard [15] have proposed that TU–C formation occurs via a thietane intermediate. This attractive mechanism is compatible with our findings: it predicts a reorientation of the TU 8 and C 13 residues during the photoreaction. However, this mechanism is not supported by luminescence studies of 4TU in solution or in tRNA [16]. In addition it cannot be accommodated with the coupling of benzo [a] pyrene at the 5th position of 1-methyl-cytosine under 254 nm irradiation as recently reported by Cavalieri and Calvin [17].

Acknowledgements

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