INTRODUCTION OF AN INTRAMOLECULAR FLUORESCENT PROBE IN *E. COLI* $tRNA_1^{Val}$

Alain FAVRE
Institut de Biologie Moléculaire, Faculté des Sciences, 9 Quai St. Bernard, Paris 5e, France

and

Moshe YANIV
Department of Biochemistry, Stanford University School of Medicine, Stanford, California, USA

Received 21 July 1971

1. Introduction

Fluorescence spectroscopy as a method to study nucleic acids has been retarded in part due to the lack of fluorescence from the common nucleosides in conditions where the RNA molecules are biologically active. Indirect approaches have involved chemical fixation or intercalation of fluorescent dyes. Yet these procedures may alter the RNA conformation, a difficulty which is overcome by the use of fluorescent nucleosides. This point is well-illustrated by the considerable amount of work concerning the structure and interactions of yeast $tRNA^{Phe}$ that have been done with the Y base fluorescence (see ref. in [1]). Other rare nucleosides such as N_4 acetylcytidine, N_7 methylguanosine [2] and ribothymidine [3] are known to fluoresce under biological conditions. These residues are present as minor constituents in transfer RNA, however their emission would not be easy to detect due to non selective absorption and (or) low yield of emission. We have indeed observed a weak fluorescence from 4 thiouridine, (4TU), a minor nucleotide present in *E. coli* tRNA [4]. The surprising fluorescent properties [5] of this residue and its use in the study of tRNA conformation will be described elsewhere.

In 1969 one of us (A. Favre) discovered a specific and quantitative photoreaction of 4 TU in *E. coli* $tRNA_1^{Val}$, which occurs in conditions where the tRNA molecule is biologically active [5, 6]. As shown by Yaniv et al. [7] it results in the cross-linking of TU in 8th position in *E. coli* $tRNA_1^{Val}$ sequence with a cytidine in 13th position. Since 4 TU is susceptible to sodium borohydride reduction as shown by Cerrutti et al. [8] we examined the reduction either of purified TU-C or of TU-C in $tRNA_1^{Val}$. Under appropriate conditions TU-C is readily reduced to $(TU\text{-}C)^{red}$, a product which strongly fluoresces in $tRNA_1^{Val}$ [9].

2. Experimental

Sodium borohydride ($NaBH_4$) was a Merck Sharp and Dohme product.

4 Thiouridine was prepared by reduction with metcaptoethanol of disulfide (Cyclo chemical company) and subsequent purifications as described elsewhere [4].

E. coli K12 $tRNA_1^{Val}$ was purified by fractionation on Serva benzoylated DEAE cellulose as described [5]. Total *E. coli* B tRNA was obtained from Schwart laboratories.

Irradiation at 335 nm (band-pass 6 nm) of tRNA samples, in sodium cacodylate buffer (0.15 M Na^+, pH 7) was performed at 25° with a Baush and Lomb monochromator equipped with an HBO 200 watt super pressure mercury lamp. The irradiation procedure has been described in detail [5, 6].

Purification of one optical density unit at 330 nm of TU-C photodimer from *E. coli* $tRNA_1^{Val}$ and some of its properties have been described [5]. Yet most of the work reported here is concerned with pure photodimer isolated from total *E. coli* tRNA. The two preparations have the same absorption spectra at neutral and acidic pH, they have the same sensitivity

toward sulfur reagents, and are similarly susceptible to sodium borohydride reduction. The purification procedure as well as some photoproduct properties will be described elsewhere.

Absorption spectra were measured with a Cary model 15 or a Beckman DG spectrophotometer. Most of the fluorescence studies were done with a Jobin Yvon spectrofluorimeter. Correction in emission and quantum yield determination was done by comparison with quinine sulfate in 1 N H_2SO_4 (Φ quinine 22 °C = 0.58 E/mole [11]. Corrected excitation and emission spectra were directly recorded with a Turner apparatus at Stanford. Very similar spectra were also obtained with a double-beam absolute spectrofluorimeter Fica model 55 in Paris.

Fluorescence time-life was measured with TRW 75 A fluorimeter equipped with a Tektronik oscilloscope.

3. Results

3.1. *$NaBH_4$ reduction of the TU-C photodimer*

The reduction was performed in conditions similar to those used for 4 thiouridine [8]: the sample in cacodylate buffer 0.05 M, NaCl 0.1 M pH 7 at 25° was brought to pH 9.7 with concentrated ammonia and its UV spectra recorded at various times after addition of $NaBH_4$ in the range 10^{-8} to 10^{-1} M.

The absorption spectra of the pure photodimer shows considerable changes after reduction. On the long wavelength side there appears a new peak at 379 nm with concomitant decrease at 330 nm. Reduction of TU-C in pure $tRNA_1^{Val}$ is followed by very similar changes (fig. 1a), a new peak now at 386 nm. The presence of an isosbestic point at 350 nm for pure TU-C (at 356 nm for $tRNA_1^{Val}$) indicates that the conversion is quantitative and that a single product is formed.

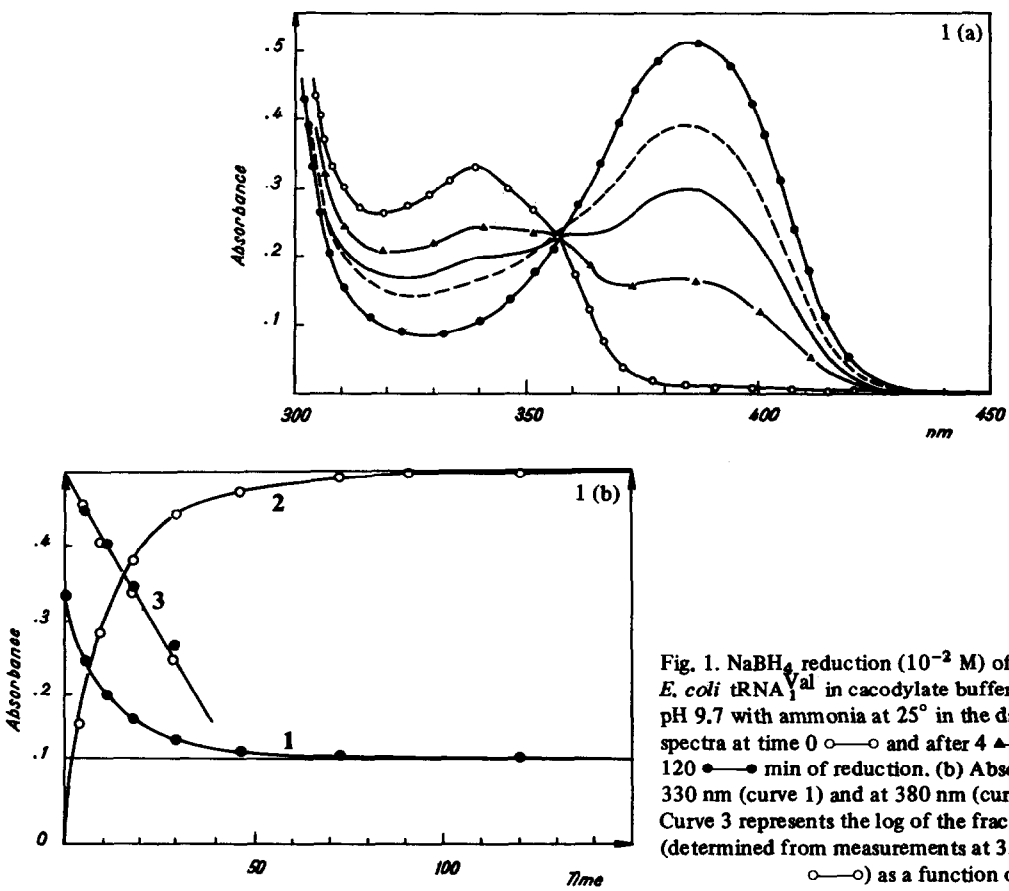

Fig. 1. $NaBH_4$ reduction (10^{-2} M) of 335 nm irradiated *E. coli* $tRNA_1^{Val}$ in cacodylate buffer. 0.15 M Na^+ brought to pH 9.7 with ammonia at 25° in the dark. (a) Absorption spectra at time 0 o—o and after 4 ▲—▲ 10 — 20 - - - - and 120 •—• min of reduction. (b) Absorption variation at 330 nm (curve 1) and at 380 nm (curve 2) during reduction. Curve 3 represents the log of the fraction of remaining TU-C (determined from measurements at 330 •—• or 380 nm o—o) as a function of time.

Table 1
Kinetics of $NaBH_4$ reduction.

	TU[a]	TU[a] (tRNA total)	TU-C	TU-C $tRNA_1^{Val}$
Initial concentration in $NaBH_4$ in M	5×10^{3}	1.25×10^{-1}	5×10^{-3}	5×10^{-3}
measured half-time in min	200	200	0.5	14
initial concentration in TU or TU-C residues in M	10^{-4}	0.6×10^{-4}	0.3×10^{-4}	0.3×10^{-4}

[a] These conditions are close to those used by Cerutti et al. [8].

$$\text{TU-C} \longrightarrow (\text{TU-C})^{red}.$$

According to this, the kinetics are the same whether observed at 340 or 380 nm (fig. 1b). They appear to be first-order and the rate constant K in the $tRNA_1^{Val}$ case is roughly proportional to the initial concentration C_0 of $NaBH_4$ ($K/C_0 = 6\ min^{-1}\ M^{-1}$).

Although it has been shown that TU is reduced to *N*-rinosyl-2-oxo-hexahydropyrimidine the kinetics have not been detailed [8]. Since the latter product no longer absorbs light in the range of 300–400 nm the reaction was followed by UV absorption and the results are summarized in table 1. It shows that if all experiments are normalized to an initial concentration of 10^{-2} M in $NaBH_4$ the half-time should be respectively 1, 28, 200 and 10^4 min for pure TU-C, TU-C in $tRNA_1^{Val}$, pure TU, and TU in total tRNA. Clearly the tRNA structure has a similar effect on the reduction rate of TU-C and TU. The most striking result is the much higher susceptibility of TU-C (400 times more than TU) to reduction. This point is of particular interest in view of tRNA specific modification.

The pure $(TU\text{-}C)^{red}$ product is unstable in the re-

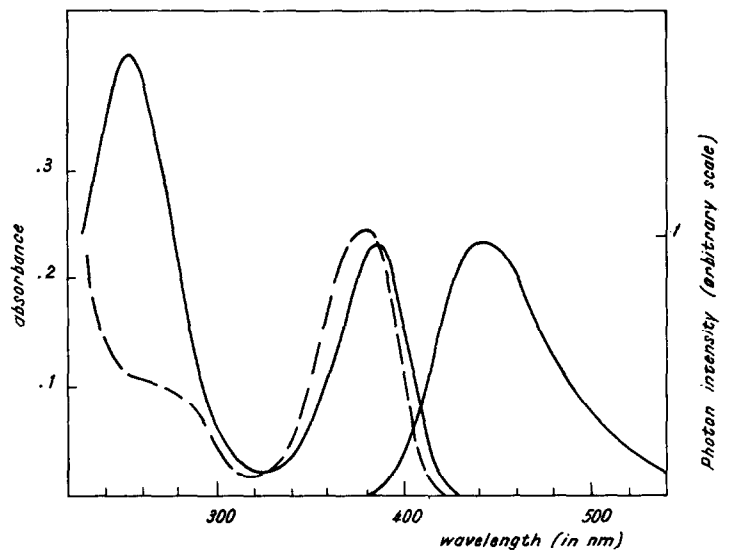

Fig. 2. Fluorescence excitation (λ em 450 nm) and emission spectra of $(TU\text{-}C)^{red}$ in $tRNA_1^{Val}$ (0.05 OD^{260} in cacodylate buffer 0.15 M Na^+, 10^{-2} M $MgCl_2$, pH 7 at 4°) as measured with the Fica 55 absolute spectrofluorimeter. The dotted line - - - - - represents the absorption spectrum of pure $(TU\text{-}C)^{red}$, in cacodylate buffer pH 7.

ducing conditions at 25°. Yet it is stable for months in tRNA at −20° in neutral solutions. The chemical nature of both TU-C and $(TU\text{-}C)^{red}$ products are under investigation.

3.2. *Finger-print study*

Finger-print studies of irradiated ^{32}P $tRNA_1^{Val}$ have shown that at least 85% of TU was of the TU-C form [7]. The same method was applied to irradiated ^{32}P $tRNA_1^{Val}$ that had been reduced with $NaBH_4$ (10^{-2} M) for 1 hr and then neutralized. It showed that the TU-C linkage remains stable during the reduction. In addition according to the kinetic studies, more than 90% of the minor $tRNA_1^{Val}$ nucleosides such as dihydrouridine and 7 methyl-guanosine, that are sensitive to $NaBH_4$ reduction [8], are recovered.

3.3. *$(TU\text{-}C)^{red}$ fluorescence*

In cacodylate-buffer (0.15 M Na^+ pH 7, 10^{-2} M Mg^{2+}) $(TU\text{-}C)^{red}$ in $tRNA_1^{Val}$ is fluorescent. Its excitation and emission spectra intersect at 415 nm. The shape of the emission spectrum (λ_{max} 440 nm) is independent of the excitation wavelength (fig. 2). The quantum yield is of 22 ± 5% at 25° and does not vary with tRNA concentration in the range 5×10^{-6} to 10^{-3} M.

Determinations of the time-life at 25° shows it is shorter than 5 nsec. Since the molar extinction coefficient of $(TU\text{-}C)^{red}$ in tRNA is high (ϵ^{385} = 20×10^4 $mole^{-1}$), the theoretical intrinsic time-life [11] is as short as 10 nsec, and the actual time-life should be of the order of 3 nsec.

The excitation spectrum shows on the long wavelength side a peak (λ_{max} 388 nm) which corresponds well with $(TU\text{-}C)^{red}$ absorption spectrum (λ_{max} 386 nm) in $tRNA_1^{Val}$. Another peak with a λ_{max} near 260 nm is observed on the short wavelength side. This peak is much higher than expected from absorption of the pure $(TU\text{-}C)^{red}$ product (fig. 2). In $tRNA_1^{Val}$ $(TU\text{-}C)^{red}$ absorption in the range of 200–300 nm may be modified. This is indicated by a hypochromicity of 35% at 380 nm after T_1 and pancreatic RNase-digestion of $tRNA_1^{Val}$. Yet an increase in absorption corresponding to the 260 nm excitation peak is not highly probable. The most likely explanation of this peak is an efficient energy transfer from the common bases to the $(TU\text{-}C)^{red}$ product.

The quantum yield of emission of $(TU\text{-}C)^{red}$ is very sensitive to alteration in the tRNA structure. Digestion in the presence of 10^{-2} M Mg^{2+} by T_1 or pancreatic ribonuclease decreases the quantum yield by a factor 3 and 15 respectively. The pure $(TU\text{-}C)^{red}$ product is practically non-fluorescent, its yield being $\frac{1}{400}$ of that of $tRNA_1^{Val}$.

4. Discussion

Irradiation at 335 nm of $tRNA_1^{Val}$ converts specifically at least 85% of TU into the TU-C photodimer [5, 6]. The finger-print study of irradiated and reduced $tRNA_1^{Val}$ showed that the reduction reaction in $tRNA_1^{Val}$ is quantitative and quite specific for TU-C, in agreement with the reduction kinetic studies. The overall process converts at least 80% of TU present in *E. coli* $tRNA_1^{Val}$ into $(TU\text{-}C)^{red}$. It is quite specific for TU since 90% of the other $NaBH_4$ sensitive nucleotides are recovered. We can then induce a covalently linked fluorescent probe in a specific position of $tRNA_1^{Val}$. This reaction is of major interest for it can be used to study tRNA conformation and interactions.

This method provides a simple and sensitive test to detect the TU-C photodimer. It has been used to measure the extent of TU-C formation in $tRNA_1^{Val}$ as a function of the medium and temperature. It allowed us to show that the TU-C cross link is highly specific for those *E. coli* tRNA species containing a TU_8 and C_{13} [5, 6]. Our method has recently been used to detect TU-C formation in altered $tRNA_1^{Val}$ and $tRNA^{fMet}$ [12, 13].

Since $(TU\text{-}C)^{red}$ fluoresces strongly in $tRNA_1^{Val}$ but much less in aqueous solvent, it certainly lies in an hydrophobic region. A more detailed study confirmed this point, and showed that the fluorescence yield is very sensitive to the divalent cation concentration, a result reminiscent of those obtained with the Y base [11]. This fluorescence has also been used for energy transfer experiments between covalently linked or intercalated dyes in tRNA.

We have previously shown that introduction of the TU-C photodimer resulted in a localized conformational alteration near TU. It also resulted in the distortion of a large portion of tRNA (some 20 nucleotides)

distal from TU [5]. The irradiated $tRNA_1^{Val}$ remains chargeable and its affinities for valine synthetase decreased by a factor of 3 [15]. Very similar observations have been done with the irradiated and reduced $tRNA_1^{Val}$ (this tRNA remains chargeable and its affinity for the synthetase is decreased by a factor of 2) leading to the same conclusion: the region of TU is not directly recognized by the enzyme. Interaction between $(TU\text{-}C)^{red}$ containing tRNA and its effectors (tRNA synthase or ribosomes...) can be more specifically studied using energy transfer between the effector's tryptophane residues and $(TU\text{-}C)^{red}$. Work along these lines is in progress.

Acknowledgements

We are indebted to Dr. Arrio Berbard for his help in time-life measurements and to Dr. H. Buc for using the Fica 55 absolute spectrofluorimeter. We also thank Prof. F. Gros and Dr. A.M. Michelson for their interest and support in this work. This work was supported by grants from the Centre National de la Recherche Scientifique, the Fond de Développement de la Recherche Scientifique et Technique, the Commissariat à l'Energie Atomique, the Lique Nationale Française contre le Cancer, and the Fondation pour la Recherche Médicale Francaise.

References

[1] K. Beardsley, T. Tao and C.R. Cantor, Biochemistry 18 (1970) 3254.
[2] F. Pochon, M. Leng and A.M. Michelson, Biochim. Biophys. Acta 199 (1968) 350.
[3] W. Hauswirth and M. Daniels, Photochem. Photobiol. 13 (1971) 157.
[4] H.N. Lipsett, Cold Spring Harbor Symp. Quant. Biol. 31 (1966) 449.
[5] A. Favre, A.M. Michelson and M. Yaniv, J. Mol. Biol. 58 (1971) 367.
[6] A. Favre, M. Yaniv and A.M. Michelson, Biochem. Biophys. Res. Commun. 37 (1969) 266.
[7] M. Yaniv, A. Favre and B.G. Barrell, Nature 223 (1969) 1331.
[8] P. Cerutti, J.W. Holt,and N. Miller, J. Mol. Biol. 34 (1968) 505.
[9] A. Favre, Structure secondaire et tertiaire des acides nucléiques, Thèse de Doctorat d'Etat, Université de Paris VII (1971).
[10] J.W. Eastman, Photochem. Photobiol. 6 (1967) 55.
[11] N.J. Turro, in: Molecular Photochemistry (Benjamin, New York, 1967).
[12] M. Krauskoff and J. Ofengand, FEBS Letters 15 (1971) 111.
[13] M.A.Q. Siddiqui and J. Ofengand, FEBS Letters 15 (1971) 105.
[14] J. Eisinger, B. Feuer and T. Yamane, Proc. Natl. Acad. Sci. U.S. 65 (1969) 638.
[15] M. Yaniv, A. Chestier, F. Gros and A. Favre, J. Mol. Biol. 58 (1971) 381.