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Measurement of the intersystem crossing yield of s4U within tRNA

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Abstract: The rare base 4-thiouridine (s4U), present in various transfer RNA (tRNA) molecule from *Escherichia coli*, occupies usually the strategically important 8-th position between the double helices of the acceptor and the dihydrouridine stems of the cloverleaf. This unusual base is largely used as an intrinsic build in probe for RNA conformational and RNA (DNA)-protein interaction studies through triplet excited state photochemistry related to covalent adducts formation. Here, by applying laser transient absorption saturation spectroscopy, we measured the intersystem crossing yield φ_{ST} and the excited triplet state absorption $\varepsilon_{T\text{-}T}$ of s4U within tRNA. While the incorporation of s4U in tRNA induced appreciable changes in the latter, no important variation of the intersystem crossing yield was observed which is in contrasts with the published data.

Keywords: *Photocrosslinks, flash photolysis, saturation, spectroscopy, fluorescent, ionization, puls energy, measuring.*

Introduction

About 70% of the different transfer RNA (tRNA) molecules content in the 8th position the rare nucleotide 4-thiouridine (s^4U). The substitution of the heavy sulphur for oxygen in position 4 of s4U result in drastic changes in the spectral properties, namely the long wavelength absorption band maximum is shifted from 260 to 330 nm (Favre, 1973; Shalitin & Feitelton, 1973). While free s4U or tRNA incorporated is not fluorescent, it exhibits at room temperature unusual emission peak at 550 nm attributed to phosphorescence from the long lived triplet state T_1 (Favre, 1973; Shalitin & Feitelton, 1973; Salet *et al*., 1983; Bensasson & Land, 1971). The values of the intersystem crossing yield φ_{ST} and other spectroscopic constants of monomer s4U have been determined by using different methods. In early works the value φ_{ST}~2 10^{-2} (Salet *et al*., 1983) was found by actinometry, using anthracene as standard and the energy transfer determined triplet-triplet extinction value ε_{520}^{TT}=5 10^4 $M^{-1}cm^{-1}$ in acetonitrile (Salet *et al*., 1983; Bensasson & Land, 1971)**.** The latter value turned out, however, to be an order of magnitude overestimated, thus leading to equivalent underestimation of φ_{ST}. By contrasts, the value φ_{ST}~1 was found in (Milder & Kliger, 1985) by transient absorption spectroscopy, which is obviously overestimated due to neglecting the triplet-triplet absorption. Finally, the most reliable value of φ_{ST}=0.67 was determined (Heihoff *et al*., 1990; Favre, 1990) by using several different techniques, as the singlet oxygen phosphorescence, laser optogalvanic spectroscopy and time resolution thermal lens.

NMR data suggest the formation of non Watson-Crick base pairs between s^4U and adenine at position 13 in tRNA (Shalitin & Feitelson, 1976). This has been directly confirmed by photochemistry showing the formation of covalent photocrosslinks between s^4U and cytosine at position 13 (Hyde & Reid, 1984; Dubreuil, 1985). Therefore, the spectroscopic properties of s^4U might be expected to vary by changes in the secondary and tertiary structures of t-RNA induced by salt and/or temperature. In deed, T_1 state lifetime increases from 200 ns to 6.6 μs in dependence of mono- and di-valent cations concentration (Favre, 1990; Shalitin & Feitelson, 1976; Leroy, 1997) leading to increase of the phosphorescence yield in a temperature dependent way.

While the shifted spectral maximum enables selective excitation of s^4U within t-RNA, the higher intersystem crossing yield is useful for efficient population of its triplet state. Although, the value of φ_{ST} for free s^4U was firmly established there is little information whether this value varies upon incorporation in t-RNA and its structural transitions. The only data (Milder et al., 1989) provided

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shows an important decrease of the intersystem crossing yield for s4U incorporated in tRNA. Here we have measured the intersystem crossing yield of s4U within t-RNA by transient absorption saturation spectroscopy and found its value similar to that for free s4U and independent on salt and temperature induced conformation changes.

Materials and Method

Products

s4U and total tRNA are from Sigma and Boeringer Mannheim respectively. All other chemicals used were obtained from Merk. All solutions were done using bi-distilled de-ionized water. To completely remove divalent cations present in the commercial sample tRNA was dissolved in water at 10 mg/ml and successively dialyzed three times at 4°C against 250 ml of 25 mM phosphate buffer, pH 7.2 - 20 mM EDTA - 0.15 M NaCl. This was followed by three 2h dialyses against 25 mM phosphate buffer, pH 7.2 - 5 mM NaCl. The dialysed stock solution was aliquoted and stored at -20°C.

The solutions concentration was adjusted by optical density readings using the following values of the molar extinctions at the maximum of the 330-335 nm absorption band: 22,000 $M^{-1}cm^{-1}$ and 14,660 $M^{-1}cm^{-1}$ for free s4U and tRNA respectively (Favre, 1990).

Laser flash-photolysis

The laser flash photolysis set-up used (figure 1) was based on the third harmonics (λ=355 nm, pulse duration τ_p=35 ns) of a Nd:YAG laser (JK) as an excitation source. The relative laser pulse energy was measured by large area UV photodiode. The transient absorption was monitored at the perpendicular direction by using the collimated beam of a pulsed Xe flash-lamp. The dimensions of the laser and the lamp beams at the entrance of the 0.5x2 cm quartz cell were adjusted to 0.3x0.3 cm and 0.25x0.1 cm respectively by using diaphragms. The temperature was set up at 20ºC by means of water circulating pump monitored by a thermocouple. The probing beam was focussed on the slit of a Spex single-grating monochromator and the signal recorded by the model R 928 Hamamatsu photomultiplier connected to a Hewlett Packard 1 Gs/s digital oscilloscope interfaced to a PC via IEEE 488 protocol.

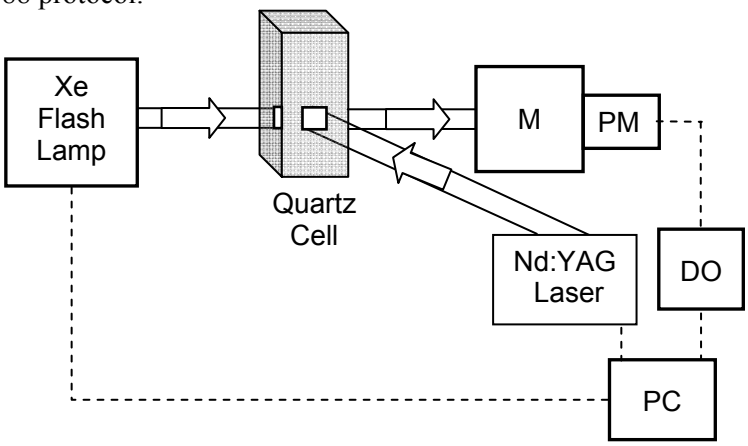

Figure 1. Scheme of the laser flash-photolysis set-up. M- monochromator, PM – photomultiplier, DO - digital oscilloscope, PC – personal computer.

Results and Discussion

An overview of the literature shows that for free s4U the value φ_{ST} =0.67±15 % is the most reliable. By contrasts, the only published value φ_{ST} =0.35 for s4U within tRNA is questionable (Milder *et al*., 1989) since excited state absorption was neglected. We have measured this value applying a direct method of ground state absorption saturation. The method consists in measuring the optical density at the end of laser pulse as a function of exciting laser intensity. For the purpose, the above described technique of nanosecond laser flash photolysis has been used.

The optical density change at the maximum of the absorption band (λ=330 nm) was calculated from the experimentally measured steady state photomultiplier current before (A) and at the end the laser pulse (ΔA) using the formula:

$$\Delta D = -\lg\left(\frac{A}{A+\Delta A}\right) \quad (1)$$

Thus, the transient optical density in a 1 cm path length is given by:

$$D = D_0 - \frac{\Delta D}{0.3} = D_0 - 3.33\lg\left(\frac{A}{A+\Delta A}\right) \quad (2)$$

D_0 is the stationary optical density of the solution measured by conventional spectrophotometer in a 1 cm path length. The coefficient 3.33 relies to the irradiated 0.3 cm layer in the direction of the probing beam.

Let analyze the reasons for the optical density change in the maximum of absorption band (λ=330-340 nm). Since the excited S_1 population is negligible due to its short lifetime ~10^{-12} s, the experimentally observed bleaching of the first absorption band around 330 nm is due uniquely to depletion of the ground S_0 state at the expense of the population of the triplet T_1 state. The lifetime of the latter is considerably longer than the laser pulse duration. By contrast to (Milder *et al.*, 1989) **we** do not neglect the triplet state absorption and consider it to play a role in the ground state bleaching. In addition, our observation of the lack of significant transient absorption from hydrated electrons at 700 nm (not shown) demonstrates that the biphotonic ionization is negligibly small. Probably the excitation energy of about ~6 eV by two 355 nm photons absorption is below the ionization limit of s4U. Therefore, the contribution of radicals generated by two-quantum mechanism is negligible in the 330-340 nm absorption.

Under these conditions in 1 cm path length:

$$D = \varepsilon_1 C_1 + \varepsilon_2 C_2 \quad (3)$$

where D is the transient optical density at the maximum of the absorption band, ε_1 and ε_2 are the molar extinction of the ground S_0 and the excited triplet T_1 state, and C_1 and C_2 are the population concentrations of these states respectively.

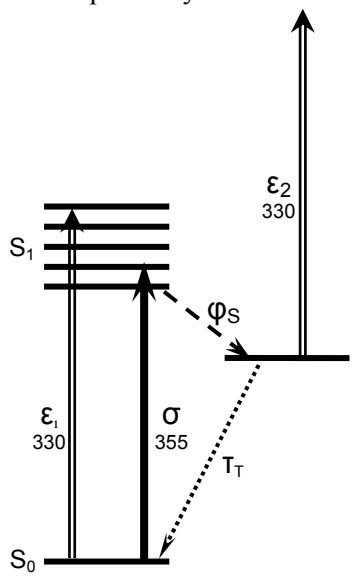

Figure 2. Simplified energy diagram used to describe the experiment. S_0 - the ground state, S_1, T_1 – the excited singlet and triplet states respectively. ε_1 and ε_2 are the molar extinction of S_1 and T_1 resp., σ – the absorption cross-section of the $S_0 \rightarrow S_1$ transition (λ=355 nm).

The rate equations for the molecule concentration in approximation of thin layer excitation are as follows:

$$\frac{dC_1}{dt} = -\varphi_{ST}\sigma C_1 I(t) \qquad t \le 0:\ I(t) = 0;\ C_1 = C_0;\ C_2 = 0 \qquad (4)$$
$$C_1 + C_2 = C_0 \qquad 0 < t \le \tau_p :\ I(t) = I$$

where: σ is the ground state absorption at 355 nm, $\varphi_{ST} = \frac{\tau_{ST}^{-1}}{\tau_{ST}^{-1} + \tau_1^{-1}}$ is the intersystem crossing yield, I(t) is the laser intensity [photons cm^{-2} s^{-1}], C_0 is the initial ground state concentration, τ_p is the laser pulse duration (35 ns), τ_1 and τ_T are the singlet and triplet state lifetimes respectively ($\tau_1 << \tau_p << \tau_T$).

The solutions of the rate equations at the end of the laser pulse $t = \tau_p$ are:

$$[S_0] = C_0 \exp(-\varphi_{ST}\sigma E)$$
$$[T_1] = C_0[1 - \exp(-\varphi_{ST}\sigma E)] \qquad (5)$$

where E is the laser pulse dose ($E = I.\tau_p$) [photons cm^{-2}].
By replacing (Milder & Kliger, 1985) into (Salet *et al*., 1983) we obtain:

$$D = D_0\left[\left(1 - \frac{\varepsilon_2}{\varepsilon_1}\right)\exp(-\varphi_{ST}\sigma E) + \frac{\varepsilon_2}{\varepsilon_1}\right] \qquad (6)$$

The experimental curves D=f(E) for s4U free and within tRNA are presented on Figure 3. Taking into account that values of $\varepsilon_1(334)$ = 22000 (14660) $M^{-1}cm^{-1}$ and $\sigma(355) = 2.88\ 10^{-17}$ ($3.44\ 10^{-17}$) cm^2 for free s4U and tRNA incorporated are known, the transient optical density depends on two unknown parameters φ_{ST} and ε_2 only. Their values have been determined by least squared fitting of experimentally determined values of D as a function of the laser pulse dose E (D = f(E), using the equation 6 (Figure 3).

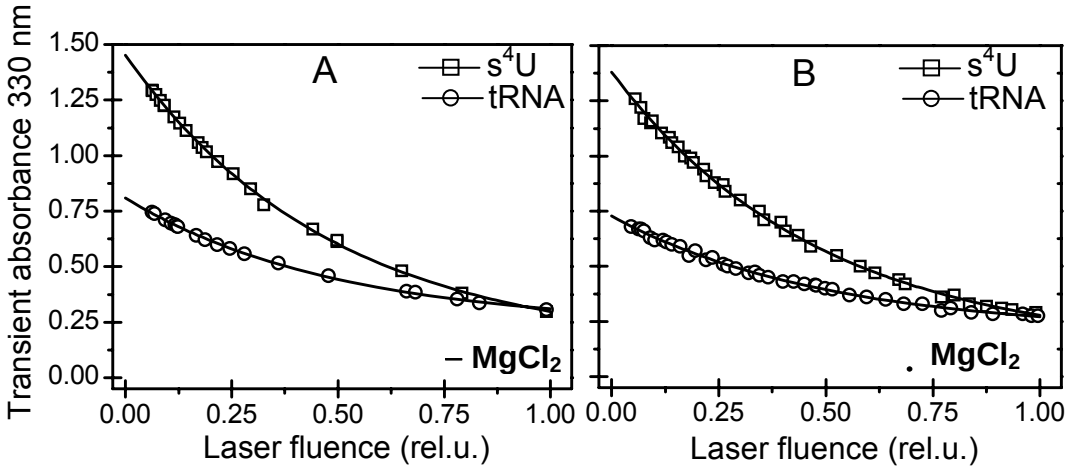

Figure 3. Transient absorbance D(334) at the end of the laser pulse versus the laser fluence E in absence (A) or presence (B) of 1.5 mM $MgCl_2$.

It should be noted that the laser pulse energy has been measured in relative units by using non-calibrated photodiode. We, therefore, used the known value φ_{ST}=0.67±15% for free s4U for calibration of the laser pulse dose. In this way we obtain $\varphi_{ST}^{tRNA} = k.\varphi_{ST}^{s4U}$, where k=0.85±10%. Therefore φ_{ST}^{tRNA}=0.57±30%. The obtained value of φ_{ST}(tRNA) is close to that of free s4U. By contrasts, the authors of (Milder *et al*., 1989) obtained k = 0.3 and φ_{ST}(tRNA)=0.33. This discrepancy with (Milder *et al*., 1989) is due to neglecting different extinction values of free and tRNA incorporated s4U in the

calculation of φ_{ST}^{tRNA} from experimental data in and importantly also in neglecting the excited triplet state absorption which is considerable for tRNA. The method of the ground state absorption saturation allowed not only to determine φ_{ST} but also to estimate the T-T excited state absorption. We obtained ε_{340}^{T-T}=1700 and 4100 $M^{-1}cm^{-1}$ for s4U free and within tRNA respectively, the error being about 10-15%. It is to be noted that experimental data on this value for s4U within tRNA are lacking in the literature. Therefore, the value of $\varepsilon_2^{T-T}/\varepsilon_1$ in the maximum of absorption band (330-340 nm) is 0.086 for free s4U and 0.30 for s4U within tRNA.

Finally, the saturation curves of tRNA do not show any changes with the addition of divalent ions that is known to induce structural changes. Therefore the value of φ_{ST} for s4U not only shows little dependence on its incorporation in tRNA but is independent also on its secondary and tertiary structure.

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