Printed ISSN 1330–0008
Online ISSN 1333–9125
CD ISSN 1333–8390
CODEN FIZAE4

MEASUREMENT OF THE INTERSYSTEM CROSSING YIELD OF s^4U WITHIN tRNA BY TIME-RESOLVED ABSORPTION BLEACHING

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Received 6 March 2008; Accepted 19 November 2008
Online 28 January 2009

The rare base 4-thiouridine (s^4U), present in various transfer RNA (tRNA) molecules 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 $\varepsilon_{\mathrm{T-T}}$ of s^4U within tRNA. While the incorporation of s^4U 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.

PACS numbers: 87.14.Gg, 87.15.Kg UDC 539.199, 539.196

Keywords: 4-thiouridine (s^4U), RNA conformational and RNA(DNA)–protein interaction, laser transient absorption saturation spectroscopy, intersystem crossing yield, excited triplet state absorption

1. Introduction

About 70% of the different transfer RNA (tRNA) molecules content in the 8^{th} position the rare nucleotide 4-thiouridine (s^4U). The substitution of the heavy sulfur for oxygen in position 4 of s^4U result in drastic changes in the spectral properties, namely the long wavelength absorption band maximum is shifted from 260 to 330

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nm [1,2]. While the free s^4U or tRNA incorporated is not fluorescent, it exhibits at room temperature an unusual emission peak at 550 nm attributed to phosphorescence from the long lived triplet state T_1 [1−4]. The values of the intersystem crossing yield φ_{ST} and other spectroscopic constants of monomer s^4U have been determined by using different methods. In early works, the value $\varphi_{\mathrm{ST}} \sim 2\ 10^{-2}$ [3] was found by actinometry, using anthracene as standard, and the energy transfer determined triplet-triplet extinction value $\varepsilon_{520}^{TT} = 5\,10^4\ \mathrm{M}^{-1}\mathrm{cm}^{-1}$ in acetonitrile [3,4]. However, the latter value turned out, to be an order of magnitude overestimated, thus leading to equivalent underestimation of φ_{ST}. By contrast, the value $\varphi_{\mathrm{ST}} \sim 1$ was found in Ref. [5] by transient absorption spectroscopy, which is obviously overestimated due to neglecting the triplet-triplet absorption. Finally, the most reliable value of $\varphi_{\mathrm{ST}} = 0.67$ was determined [6,7] 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 [8]. This has been directly confirmed by photochemistry showing the formation of covalent photocrosslinks between s^4U and cytosine at position 13 [9,10]. Therefore, the spectroscopic properties of s^4U might be expected to vary by changes in the secondary and tertiary structures of tRNA induced by salt and/or temperature. Indeed, T_1 state lifetime increases from 200 ns to 6.6 μs, depending on mono- and di-valent cation concentration [7,8,11], leading to an increase of the phosphorescence yield in a temperature-dependent way.

While the shifted spectral maximim enables selective excitation of s^4U within tRNA, 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 tRNA and its structural transitions. The only data [12] provided show an important decrease of the intersystem crossing yield for s^4U incorporated in tRNA. Here we have measured the intersystem crossing yield of s^4U within tRNA by transient absorption saturation spectroscopy and found its value similar to that for free s^4U and independent on salt and temperature-induced conformation changes.

2. *Experimental section*

2.1. *Products*

s^4U and total tRNA used in the measurements are from Sigma and Boeringer Mannheim, respectively. All other chemicals were obtained from Merck. All solutions were prepared 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 solution 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: 22000 $M^{-1}cm^{-1}$ and 14660 $M^{-1}cm^{-1}$ for the free s^4U and tRNA, respectively [7].

2.2. Laser flash-photolysis

The laser flash photolysis set-up used (Fig. 1) was based on the third harmonic ($\lambda = 355$ nm, pulse duration $\tau_p = 35$ 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 $0.5\,\mathrm{cm} \times 2\,\mathrm{cm}$ quartz cell were adjusted to $0.3\,\mathrm{cm} \times 0.3\,\mathrm{cm}$ and $0.25\,\mathrm{cm} \times 0.1\,\mathrm{cm}$, respectively, by using diaphragms. The temperature was set up at 20°C 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 Gb/s digital oscilloscope interfaced to a PC via the IEEE 488 protocol.

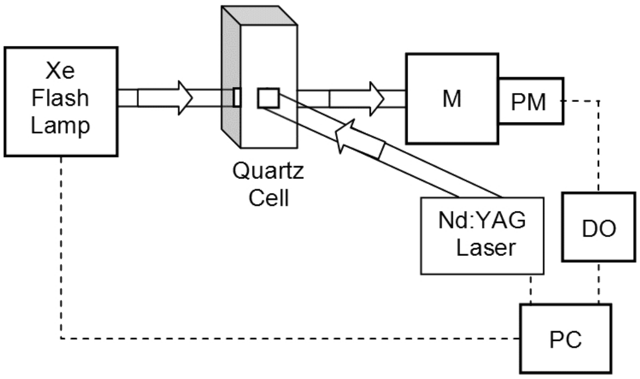

Fig. 1. Scheme of the laser flash-photolysis set-up. M - monochromator, PM - photomultiplier, DO - digital oscilloscope, PC - personal computer.

3. Results and discussion

An overview of the literature shows that for free s^4U the value $\varphi_{\mathrm{ST}} = 0.67 \pm 15$ % is the most reliable. By contrasts, the only published value $\varphi_{\mathrm{ST}} = 0.35$ for s^4U within tRNA is questionable [12] 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 ($\lambda = 330$ nm) was calculated from the experimentally measured steady state photomultiplier current before (A) and at the end the laser pulse (ΔA) using the formula

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

Thus, taking into account the 0.3 cm irradiated pathlength in the direction of the probing beam (see the irradiation geometry in Fig. 1), the transient optical density in a 1 cm pathlength is given by

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

where D_0 is the initial optical density of the solution measured by a conventional spectrophotometer for the 1 cm path length.

Let us analyze the reasons for the optical density change in the maximum of absorption band $\lambda = 330 - 340$ nm (see Fig. 2). Since the excited S_1 population is negligible due to its short lifetime of about 10^{-12} s, the experimentally observed bleaching of the first absorption band around 330 nm is due uniquely to the 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 Ref. [12], 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 s^{4}U. Therefore, the contribution of radicals generated by two-quantum mechanism is negligible in the $330-340$ nm region of absorption.

Under these conditions for the 1 cm pathlength

$$D = \varepsilon_1 C_1 + \varepsilon_2 C_2 \tag{3}$$

where D is the transient optical density at the maximum of the absorption band, ε_1 and ε_2 are the molar extinctions 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.

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

$$\begin{aligned} &\frac{\mathrm{d}C_1}{\mathrm{d}t} = -\varphi_{\mathrm{ST}}\sigma C_1 I(t), \qquad t \le 0, \qquad I(t) = 0, \quad C_1 = C_0,\ C_2 = 0, \\ &C_1 + C_2 = C_0, \qquad\qquad 0 < t \le \tau_p, \quad I(t) = I, \end{aligned} \tag{4}$$

where σ is the ground state absorption at 355 nm, $\phi_{\mathrm{ST}} = \tau_{\mathrm{ST}}^{-1}/(\tau_{\mathrm{ST}}^{-1} + \tau_1^{-1})$ is the intersystem crossing yield, $I(t)$ is the laser intensity (photons/(cm^{2}s)), 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 ($\tau_1 \ll \tau_p \ll \tau_T$) [1, 12].

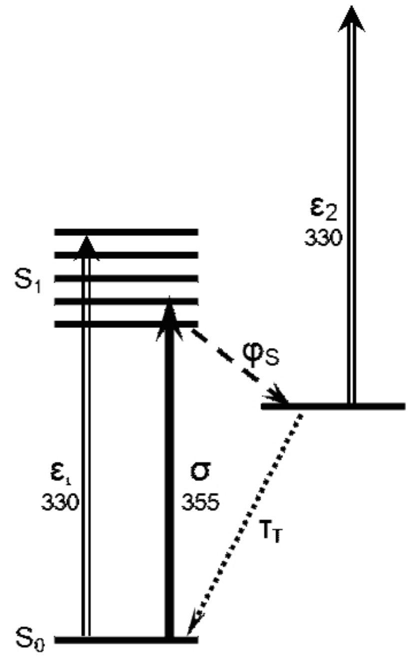

Fig. 2. Simplified energy diagram used to describe the experiment. S_0 - the ground state, S_1, T_1 *- the excited singlet and triplet states, respectively. ε_1 and ε_2 are the molar extinctions of* S_1 *and* T_1*, respectivey, σ - the absorption cross-section of the* $S_0 \rightarrow S_1$ *transition*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

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

$$\begin{aligned} \Big[S_0\Big] &= C_0 \exp(-\phi_{\mathrm{ST}}\sigma E) \\ \Big[T_1\Big] &= C_0 \Big[1 - \exp(-\phi_{\mathrm{ST}}\sigma E)\Big] \end{aligned} \tag{5}$$

where E is the laser pulse dose ($E = I\,\tau_p$) (photons/cm^{-2}). By replacing (5) into (3) we obtain

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

The experimental curves $D = f(E)$ for s^{4}U free and within tRNA are presented in Fig. 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 s^{4}U and tRNA-incorporated are known, the transient optical density depends on two unknown parameters $\varphi_{\rm ST}$ and ε_2 only. Their values have been determined by least squared fitting to experimentally determined values of D as a function of the laser pulse dose E ($D = f(E)$), using Eq. (6). The experimental curves $D = f(E)$ at zero delay time from the bleaching pulse for s^{4}U, free and within tRNA, are presented in Fig. 3.

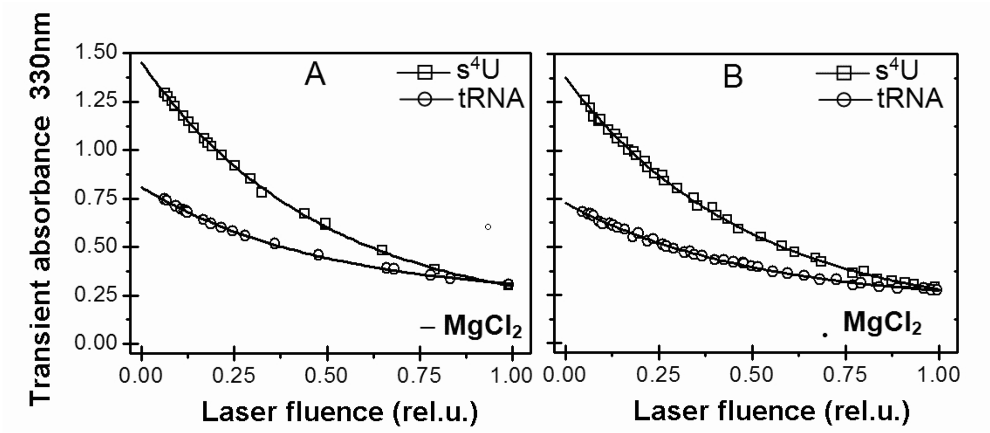

Fig. 3. Transient absorbance $D(334)$ *at the end of the laser pulse versus the laser fluence* E *in the absence* (A) *or presence* (B) *of* 1.5 mM $\mathrm{MgCl_2}$.

It should be noted that the laser pulse energy has been measured in relative units by using non-calibrated photodiode. Therefore, we used the known value $\varphi_{\rm ST} = 0.67 \pm 15\,\%$ for free s^{4}U for calibration of the laser pulse dose. In this way we obtain $\varphi_{\rm ST}^{\rm tRNA} = k\,\varphi_{\rm ST}^{\rm s^4U}$, where $k = 0.85 \pm 10\,\%$. Therefore, $\varphi_{\rm ST}^{\rm tRNA} = 0.57 \pm 30\,\%$. The obtained value of $\varphi_{\rm ST}^{\rm tRNA}$ is close to that of free s^{4}U. By contrasts, the authors of Ref. [12] obtained $k = 0.3$ and $\varphi_{\rm ST}^{\rm tRNA} = 0.33$. This discrepancy with Ref. [12] is due to the neglecting of different extinction values of free and tRNA-incorporated s^{4}U in the calculation of $\varphi_{\rm ST}^{\rm tRNA}$ from experimental data in Ref. [12] 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 $\varphi_{\rm ST}$, but also to estimate the T-T excited state absorption. We obtained $\varepsilon_{340}^{\rm T-T} = 1700$ and 4100 $\mathrm{M^{-1}cm^{-1}}$ for s^{4}U free and within tRNA, respectively, the error being about $10-15\,\%$. It is to be noted that experimental data on this value for s^{4}U within tRNA are lacking in the literature. Therefore, the value of $\varepsilon_2^{\rm T-T}/\varepsilon_1$ in the maximum of absorption band (330-340 nm) is 0.086 for free s^{4}U and 0.30 for s^{4}U 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 $\varphi_{\rm ST}$ for s^{4}U not only shows little dependence on its incorporation in tRNA, but is independent also on its secondary and tertiary structure.

4. Conclusion

Using laser transient absorption spectroscopy, we have shown that the intersystem crossing yield of the rare nucleoside s^4U remains unchanged when it is incorporated in tRNA as well as under divalent ion induced folding of the latter. This finding is useful in designing experiments that use the s^4U as a built-in probe for 3D analysis of RNA and ribonucleic complexes.

Acknowledgements

This investigation was partly supported by the Ministry of Education and Science, Bulgaria - the National Fund for Science (grant N 1402/2004).

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MJERENJE PRINOSA MEĐUSUSTAVNOG PRIJELAZA s^4U U tRNA VREMENSKI-RAZLUČENIM IZBJELJIVANJEM

Rijetka baza 4-thiouridine (s^4U), koju nalazimo u raznim prijenosnim molekulama RNA (tRNA) Escherichie coli, obično uzima strateški važan 8. položaj između dviju zavojnica primatelja i dihidrouridinskih stapki lista djeteline. Ta se neobična baza najviše rabi kao unutarnja ugradbena proba za proučavanje interakcija konformalne RNA s RNA(DNA)–proteinima preko fotokemije tripletnog uzbuđenog stanja, povezanog sa stvaranjem kovalentnih adukata. U ovom smo radu primjenom laserske prijelazne apsorpcijske spektroskopije sa zasićenjem mjerili prinos međusustavnih prijelaza φ_{ST} i apsorpciju tripletnog stanja $\varepsilon_{\mathrm{T-T}}$ s^4U u tRNA. Dok ugrađivanje s^4U u tRNA uzrokuje priličnе promjene u tRNA, nismo opazili promjene prinosa međusustavnih prijelaza, što nije u skladu s objavljenim rezultatima.