4-Thiouridine, a Built-In Probe for Structural Changes in Transfer RNA†

N. Shalitin and J. Feitelson*

ABSTRACT: The luminescence of an aqueous solution of 4-thiouridine was compared with its emission when forming part of the polynucleotide chain of tRNA. In both cases excitation into the last absorption band at 335 nm yields a weak emission in the 520–550 nm region. However, while in aqueous solution this emission has a lifetime of ~240 ns, it increases in native tRNA to $\tau \simeq 6.6$ μs. Oxygen and Cl^- ions quench the thiouridine emission efficiently in aqueous solution while Na^+ and Mg^{2+} ions have no influence on it. On the other hand thiouridine which forms part of a tRNA molecule is quite insensitive to Cl^- ions and to O_2 while its emission is greatly enhanced by Na^+ and Mg^{2+} ions. From these salt effects as well as from data on the temperature dependence of the emission yield and the decay curve, it is concluded that the site of the thiouridine residue is very well protected within the tertiary structure of tRNA. Both permanent changes in the secondary and in the tertiary structures of the polynucleotide as well as dynamic conformation changes can be observed by following the emission characteristics of its thiouridine residue.

The base 4-thiouridine (4-tU)[1] is present in various transfer RNA molecules obtained from *Escherichia coli* (Lipsett, 1965a,b; Lipsett and Doctor, 1967). It is found at position 8 from the 5′ end of the molecule (Zachau, 1969; Barrell and Sanger, 1969) and, thus, occupies a strategically important location between the double helices of the acceptor and the dihydrouridine stems of the tRNA clover leaf as suggested by Holly (Holly et al., 1965). X-Ray diffraction data (Kim et al., 1973, 1974) show the eighth base (in $tRNA^{Phe}$) to lie between the two major double helical regions which appear in their model. Furthermore, NMR data suggest the formation of a non-Watson–Crick base pair between 4-tU at position 8 and adenine at position 14, thus taking part in the tertiary structure of the molecule (Wong and Kearns, 1974; Wong et al., 1975). The above x-ray data show that such a hydrogen bond is indeed possible. Also, the 4-tU residue has been shown to form photodimers with cytidine in position 13 (Pochon et al., 1971; Favre et al., 1971, 1972), which again indicates its proximity to the dihydrouridine loop. The spectroscopic properties of 4-tU might therefore be expected to be influenced by changes in the secondary and in the tertiary structures of the tRNA molecule.

4-tU has a characteristic absorption band at 335 nm (Lipsett and Doctor, 1967) and a room temperature emission at 550 nm (Pochon et al., 1971). The emission properties of 4-tU in solution have been described in a previous study (Shalitin and Feitelson, 1973). Briefly, it was found that the 550-nm emission has a lifetime of about 250 ns and that it comes from a triplet state. In liquid solution this emission is quenched by oxygen with a diffusion-controlled rate constant of 6×10^9 M^{-1} s^{-1}. In a low-temperature glass, two emissions with different lifetimes were observed, emissions which come from two different triplet states of 4-tU. The excitation spectrum showed that both emissions derive ultimately from the excited singlet of 4-tU and it was proposed that one of them is the ordinary triplet of 4-tU while the other is formed by a rapid first-order process either directly from the singlet or from the above triplet state of 4-tU. Some of these findings have since been confirmed by Favre (1974) who also described the quenching of the 4-tU emission by halogen and by paramagnetic ions.

When 4-tU forms part of the tRNA molecule, it has been found that in the presence of Na^+ and Mg^{2+} ions its long wavelength emission intensity increases appreciably (Pochon et al., 1971). On the other hand it is known that the physical properties of biologically active tRNA depend upon the presence of Na^+ and Mg^{2+} ions (Henley et al., 1966; Lipmann, 1969; Fresco et al., 1966; Cole et al., 1972). The temperature dependence of both the absorption and emission of the 4-tU residue seems to reflect conformational changes in the tRNA molecule (Pochon et al., 1971; Seno et al., 1969; Pochon and Cohen, 1972; Dourlent et al., 1971). In view of the available information, it seemed of interest to compare the following emission properties of 4-tU in solution and as a constituent of the tRNA molecule, properties which in one way or another reflect some structural features of tRNA: (1) the emission wavelength, quantum yield, and lifetime as a function of Na^+ and Mg^{2+} concentration and temperature (a detailed study of these variables might in part reveal the mechanism of the so-called "melting" of tRNA); (2) the quenching rate of the 4-tU emission by oxygen and by Cl^- ions (this should yield information on the rigidity and/or the dynamics of conformational changes (Lakowicz and Weber, 1973; Saviotti and Galley, 1974) of the structure encasing the 4-tU residue in t-RNA); (3) the quantum yield and lifetime of the emission for the two systems in solution and in a low-temperature glass (these data again may indicate the feasibility of dynamic conformation changes in tRNA as will be described in the Discussion).

Experimental Methods

Partially purified tRNA, from *E. coli* w, was obtained by courtesy of Mr. Ehud Ziv of our biochemistry department. Two preparations, both of which had undergone separation on BD-cellulose, were used in this study. One was more than 90% pure $tRNA_1^{Val}$ and had a biological activity of 50%. The other had an overall activity of 80%, about equally divided between

† From the Department of Physical Chemistry, The Hebrew University, Jerusalem, Israel. *Received July 22, 1975.* A preliminary report appeared in: Proceedings on Excited States of Biological Molecules, Lisbon, April 1974, Birks, J., Ed., New York, N.Y., Wiley.

[1] Abbreviations used: 4-tU, 4-thiouridine; BD-cellulose, benzoylated diethylaminoethylcellulose.

four tRNA species active towards valine, methionine, alanine, and glycine. Highly purified $tRNA^{Val}$ was obtained from Boehringer (Mannheim).

The preparations were freed from salts by dialysis against neutral EDTA (0.01 M) in presence of 0.1 M NaCl and subsequent dialysis against triply distilled water. The absorbance ratio in water at 260 and 340 nm of the final preparation was $OD_{340}/OD_{260} \simeq 0.03$ for the $tRNA_1^{Val}$, 0.022 for the tRNA mixture, and 0.0217 for the Boehringer preparation.

All measurements were carried out at a tRNA concentration of $\sim 2 \times 10^{-5}$ M ($A_{340} = 0.15$–0.3). The chemicals used were of analytical grade. Water was triply distilled and ethylene glycol was of spectroscopic grade. Emission spectra were measured in a standard fluorimeter consisting of a 250-W Xe lamp, two Bausch & Lomb 500-nm focal length monochromators and an EMI 6256 S photomultiplier. Quantum yields were measured by comparison with quinine bisulfate fluorescence (Calvert and Pitts, 1966) which was also used to correct the measured spectra (Berlman, 1965). Emission decay curves were determined by exciting with a (10 ns) nitrogen laser (Avco, Everett) (λ_{ex} 337 nm) and feeding the signal directly into a Tektronix 545 oscilloscope (Shalitin and Feitelson, 1973).

Temperature dependences were measured either in a thermostated cell or, at low temperatures, in a quartz Dewar flask fitted with three-way double windows and a copper-block cell holder.

Results

The emission quantum yield of the 4-tU residue in a salt-free tRNA solution is similar to the corresponding value for free 4-tU ($\phi \simeq 3 \times 10^{-4}$). Its room temperature emission peak lies between 550 and 560 nm, similar to the emission band of free 4-tU at ~550 nm. In both cases the excitation spectrum of this emission coincides with the long wavelength absorption band of the 4-tU moiety at 335 nm. Though the spectral properties of the above two 4-tU systems are similar, the effects of salts on these properties are dramatically different.

Effects of Salts and of Oxygen on the Emission. In the following experiments the preparation of pure $tRNA^{Val}$ was used since particularly the melting curves, described in the next paragraph, might well differ for different tRNA species. The pH of all aqueous solutions was in the range pH 5.5–7.2.

NaCl and $MgCl_2$ quench the emission of thiouridine in solution in a bimolecular reaction, the Stern–Volmer constant for NaCl being $k_{SV} = 125\ M^{-1}$, which corresponds to a deactivation rate constant of $k_d = 8 \times 10^8\ M^{-1}\ s^{-1}$. Since both CH_3COONa and $(CH_3COO)_2Mg$ do not affect the 4-tU emission (k_{SV} is close to zero), the quenching can be attributed to the Cl^- ions only and it can be concluded that Na^+ and Mg^{2+} do not influence its emission. On the other hand the emission of the 4-tU residue incorporated in tRNA increases drastically upon addition of NaCl and $MgCl_2$ as shown in Figure 1a. It is seen that, when NaCl is added to an aqueous solution of tRNA until its concentration reaches 0.1 N, the emission increases by a factor of 2.5. A further addition of $MgCl_2$ (10^{-3} M) causes an additional increase in the emission by a factor of two to three. The final solution, therefore (0.1 N NaCl, 10^{-3} M $MgCl_2$), has a quantum yield about six times larger ($\phi \simeq 2 \times 10^{-3}$) than that of free 4-tU in a salt-free solution. Since similar results were obtained by sodium and magnesium acetate as by the corresponding chlorides, it can be concluded that in the case of tRNA the influence of Cl^- ions is negligibly small which agrees with the data of Favre (1974). In separate quenching experiments, it was indeed found that

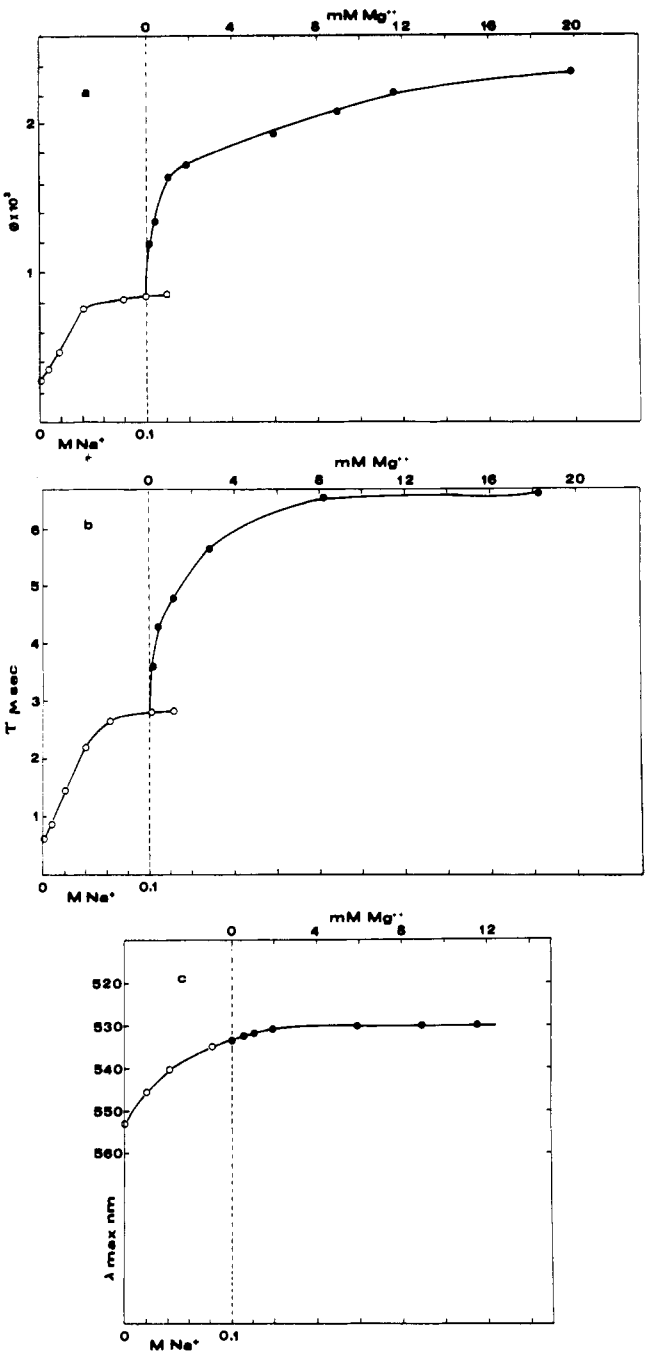

FIGURE 1: Emission properties of 4-tU in $tRNA^{Val}$; dependence on Na^+ and Mg^{2+} concentration. Na^+ was added as sodium acetate (no buffer), or as NaCl in 0.01 N Tris buffer, pH 7.2. Mg^{2+} was added as magnesium acetate (at 0.1 M sodium acetate), or as $MgCl_2$ (at 0.1 M NaCl). (a) Quantum yield of emission; excitation at 340 nm; (b) lifetime of emission; excitation by laser at 337 nm; (c) wavelength of maximal emission. Dashed vertical line separates sodium and magnesium regions.

the quenching rate constant of Cl^- ions for 4-tU in tRNA is $k_d \simeq 2.3 \times 10^5\ M^{-1}\ s^{-1}$.

It is seen from Figure 1b that, in parallel with the quantum yield, the lifetime of the excited 4-tU in tRNA increases from about 600 ns to a value of 6.6 μs upon addition of Na^+ and of Mg^{2+} ions. The corresponding value for thiouridine in a pure aqueous solution is 240 ns and decreases upon addition of Cl^- ions in accordance with the above mentioned quenching of 4-tU in solution.

It was found that the total effect on yield and lifetime is the same irrespective of whether the concentration of Na^+ ions at which Mg^{2+} is added is 0.01, 0.1, or 0.2 N.

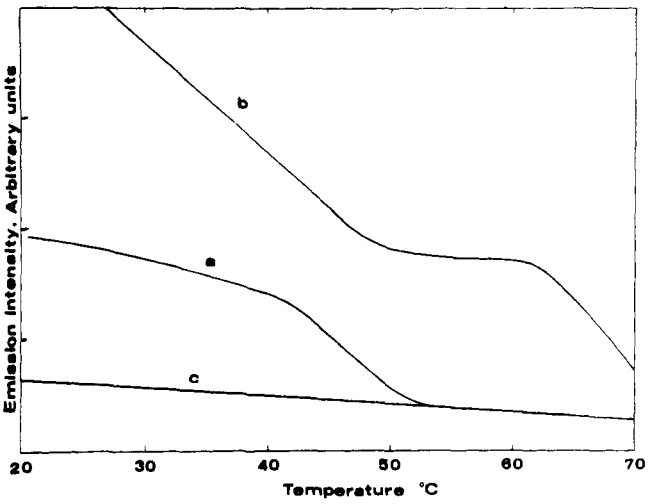

FIGURE 2: Temperature dependence of $tRNA_1^{Val}$ emission (a) in presence of 0.1 M sodium acetate, (b) in presence of 10^{-2} M sodium acetate [3×10^{-3} M $Mg(CH_3COO)_2$], (c) temperature dependence of 4-tU in water. (The slightly lower melting temperatures in our work when compared with those of Seno et al. are probably due to the lower overall salt concentrations which we used.)

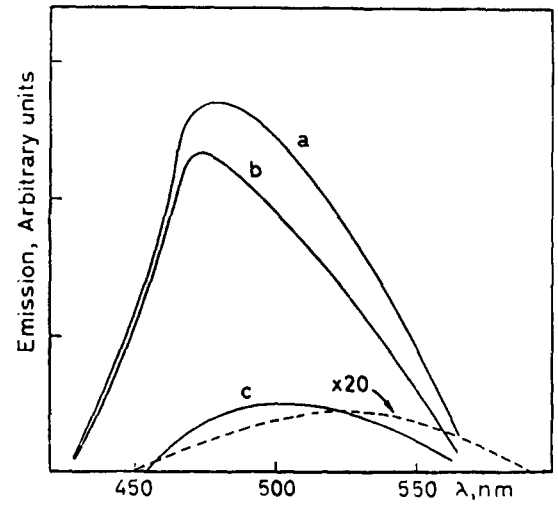

FIGURE 3: Emission spectrum of tRNA excited at 335 nm in ethylene glycol-water glass at 77 K. (a) Overall spectrum; (b) long-lived component ($\tau_1 = 2.6$ ms); (c) short-lived component ($\tau_2 = 0.6$ ms); broken line shows emission in aqueous solution at room temperature (~20 °C).

Finally, Figure 1c shows that the peak of the emission intensity over the range of Na^+ concentrations between 0.0 and 0.1 N is blue shifted by about 25 nm. No such effect is observed for free 4-tU in solution. Contrary to the effect on the quantum yield, the subsequent addition of Mg^{2+} ions does not influence the location of the peak any more. A further blue shift of the peak can be achieved upon cooling a solution of tRNA in an ethylene glycol-H_2O mixture down to 77 K, as described in the section on low-temperature measurements.

Although oxygen decreases the quantum yield of the emission of thiouridine in solution with a diffusion-controlled deactivation rate constant of $k_d = 6 \times 10^9$ M^{-1} s^{-1} (Shalitin and Feitelson, 1973), the effect on 4-tU which forms part of a tRNA molecule is much smaller. The value of the quenching constant in this case is only $k_d \leq 1.6 \times 10^7$ M^{-1} s^{-1}. No measurable decrease in the decay rate was observed in the presence of oxygen, as can be expected for such a small value of k_d.

Effect of Temperature on the Emission. (a) The 10-70 °C Range. Measurements were carried out at 3×10^{-3} M magnesium acetate in the presence of either 10^{-2} M sodium acetate or 0.1 N NaCl with 0.01 M Tris buffer, pH 7.2, added. The pure $tRNA^{Val}$ was used.

The emission of the free base 4-tU in aqueous solution is not influenced by the presence of the above buffers. Between 20 and 70 °C it decreased uniformly by about 50%, while the emission of 4-tU which formed part of the tRNA chain behaved quite differently. To begin with its quantum yield was much greater as described in the preceding section. Between 10 and 45 °C the emission intensity decreased by more than 50%. It then leveled off so that no change in emission occurred between 45 and 60 °C. Raising the temperature still further caused a sharp decrease in emission until it reached the level of free 4-tU at about 75 °C. The inflection in this curve or so-called "melting temperature" occurred at 65 °C. It can be compared with corresponding absorbance data in the literature (Seno et al., 1969; Dourlent et al., 1971).

In the presence of 0.1 N NaOAc only, i.e., with no Mg^{2+} ions added, the above sharp decrease occurred at ~45 °C (see Figure 2).

(b) The +20 to −196 °C Range. In these experiments the preparation containing four tRNA species was used. (See Experimental Methods.) Measurements of the emission in this range were carried out in an ethylene glycol-water (1:1) mixture in order to obtain a clear glass at low temperature. For the same reason the lower salt concentrations of 10^{-2} M sodium acetate and 3×10^{-3} M magnesium acetate were used which still are sufficient for the tRNA to be in its active conformation.

The emission intensity of the 4-tU residue in tRNA increases with decrease of temperature from a quantum yield of $\phi \simeq 2 \times 10^{-3}$ at 22 °C to about $\phi \simeq 0.2$ at 77 K (Figure 3). For free 4-tU in solution the quantum yield at liquid-air temperature has a similar value ($\phi \sim 0.16$), while the yield at 22 °C is almost an order of magnitude smaller ($\phi \simeq 3 \times 10^{-4}$). Furthermore, two distinct emission peaks at ~530 and at 475 nm, having different lifetimes, were observed for free 4-tU in the low-temperature glasses, while in tRNA a single peak is observed. This peak shifts gradually with decreasing temperature from ~530 nm at 22 °C to ~480 nm at 77 K. When, however, the lifetime of this emission is measured at the short and at the long wavelength edges of the peak, two distinctly different decay times can be resolved. In liquid air (77 K) at 455 nm, a purely exponential decay of $\tau = 2.6 \pm 0.1$ ms was measured (Figure 4a). At longer wavelength at this same temperature, a second short-lived component appeared in the decay curve. At the long wavelength edge of the emission peak, the short decay predominated, though some of the long-lived component still prevailed. Similar to previous data in free 4-tU, it appears that the two different species decay independently. The two lifetimes were resolved as follows. If two independent decay processes take place in tRNA, the time dependence of the emission is described by

$$I_\lambda(t) = \alpha_\lambda e^{-t/\tau_1} + \beta_\lambda e^{-t/\tau_2} \quad (1)$$

where $I_\lambda(t)$ denotes the emission intensity at time t, and α_λ and β_λ represent the initial emission of the two excited species with lifetimes τ_1 and τ_2. In our case τ_1, the lifetime at the short wavelength, is known since it could be measured directly. At long times ($t \gtrsim \tau_1$) only the long-lived component τ_1 contributes to the emission and hence in the long-time limit $\log I_\lambda(t)$ should decrease linearly, with slope $1/\tau_1$, as a function of time. A straight line with the *known* slope of $1/\tau_1$ was fitted to the long-time edge of the $\log I_\lambda(t)$ decay curve and the value of log

Table I: Emission Properties of 4-Thiouridine.

	Monomer in Aqueous Soln	As a Constituent of the tRNA Chain[a]
Quenching by Cl^-	$k_{Cl^-} = 7 \times 10^8\ M^{-1}\ s^{-1}$	$k_{Cl^-} = 2.3 \times 10^5\ M^{-1}\ s^{-1}$
by O_2	$k_{O_2} = 6 \times 10^9\ M^{-1}\ s^{-1}$	$k_{O_2} \leq 1.6 \times 10^7\ M^{-1}\ s^{-1}$
Influence on quantum yield of[b] Na^+ (0.1 M)	None	Increase × 2.5
of (addit.) Mg^{2+} (3×10^{-3} M)	None	Further increase × 2-3
Lifetimes τ		
At 20 °C	240 ns	6.6 μs
At −196 °C[c]	0.4 ms, 1.0 ms	0.6 ms, 2.6 ms
Quantum yield ϕ		
At 20 °C	3×10^{-4}	2×10^{-3}
At −196 °C[c]	~0.05 (τ = 0.4 ms)	0.05 (τ = 0.6 ms)
	~0.10 (τ = 1.0 ms)	0.15 (τ = 2.6 ms)

[a] In the presence of 10^{-2} M Na^+ and 3×10^{-3} M Mg^{2+}, except for *b*. [b] Relative to a salt-free aqueous solution. [c] In ethylene glycol-water (1:1, v/v).

α_λ was determined from its intercept at time zero (Figure 4b). By subtracting $\alpha_\lambda e^{-t/\tau_1}$ from $I_\lambda(t)$ and plotting log $(I_\lambda(t) - \alpha_\lambda e^{-t/\tau_1})$ against t, τ_2 was obtained from the slope and log β_λ from the intercept of the straight line. By repeating the procedure for different wavelengths, the same value of τ_2 should always be obtained if indeed the decay curve is described by two exponentials. This was found to be the case within the experimental error range, and a lifetime of $\tau_2 = 0.6 \pm 0.06$ ms at 77 K was obtained. The ratio of the initial emissions from the two states, of different lifetimes, can be obtained from the difference of the two intercepts log α − log β = log (α/β) in the above plot (see Figure 4b). On the other hand, integration from time zero to infinity of eq 1

$$\int_0^\infty I_\lambda(t)\mathrm{d}t = \alpha_\lambda\tau_1 + \beta_\lambda\tau_2 = F(\lambda) \qquad (2)$$

yields the intensity in a certain wavelength interval (λ to ($\lambda + \Delta\lambda$)), a quantity which is also directly obtained from the steady-state emission spectrum $F(\lambda)$. A knowledge of the quantities α/β, τ_1, and τ_2 from decay curves at different wavelengths, λ, as well as of $\alpha\tau_1 + \beta\tau_2$, as obtained from the emission spectrum, allows us therefore to separate this spectrum into its two components (see Figure 3). From these separated spectra it is seen that, in a solution containing 0.01 N Na^+ and 3×10^{-3} N Mg^{2+} ions, the long wavelength emission peak is shifted toward the blue upon lowering the temperature to 77 K.

The various emission properties of 4-tU in solution and for a 4-tU residue in the polynucleotide chain of tRNA are summarized in Table I.

Discussion

Lately the highly sensitive NMR method has been developed to probe specifically the various base pairs in tRNA for their structure and for structural changes in their vicinity (Kearns et al., 1973; Shulman et al., 1973a,b). The luminescence emission described here might supplement this information in that it reflects changes in the neighborhood of a base which is hydrogen bonded in a nonconventional manner (Wong and Kearns, 1974; Wong et al., 1975) but is located between two sequences of Watson-Crick base pairs in a region known to have a tertiary structure (Kim et al., 1973, 1974).

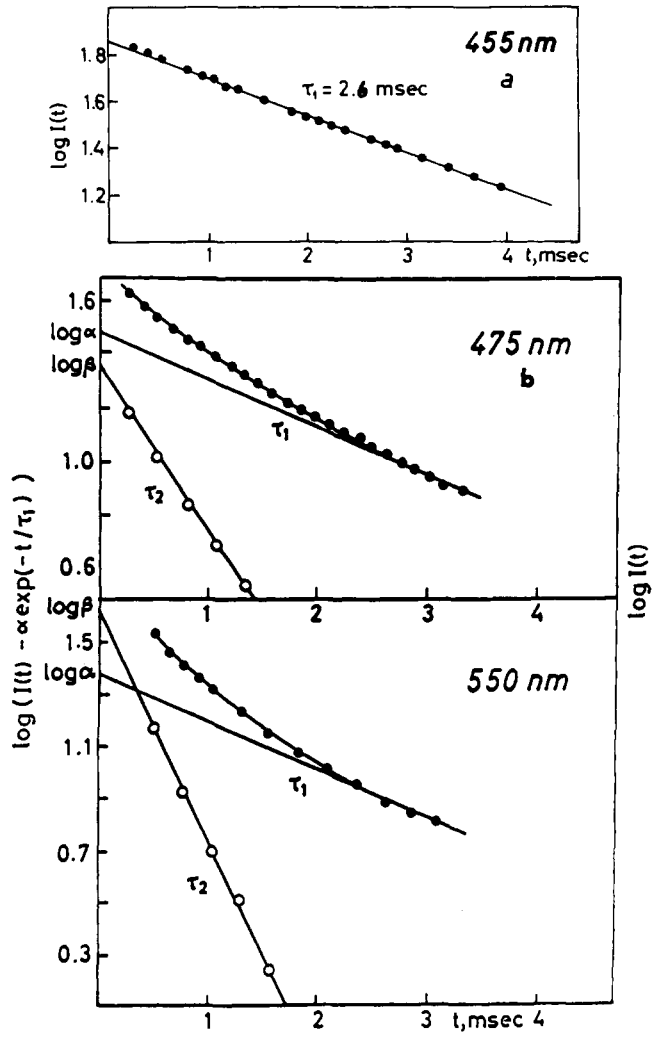

FIGURE 4: Separation of components from lifetime measurements. (a) Experimental value of log $I(t)$ is shown by full circles. (b) Log $[I(t) - \alpha \exp(-t/\tau_1)]$ refers to the open circles only. The slope of the straight line (τ_1) is taken from measurements at λ 455 nm where $\beta = 0$.

From the hypochromicity at 260 and at 340 nm and from its temperature dependence it is known that in the presence of Na^+ ions the base-paired regions of tRNA form double helical structures (Seno et al., 1969). The addition of Mg^{2+} ions, which are necessary for tRNA to be biologically active, does not cause an appreciable additional hypochromic effect. However, the addition of Mg^{2+} ions is probably accompanied by the formation of a stabilizing, tertiary structure, since the temperature at which the double helical structure disappears is raised in the presence of Mg^{2+} ions.

The dependence of the 4-tU emission in tRNA on the Na^+ and Mg^{2+} ion concentrations is more complex than the absorption data. Our reference state is a solution of tRNA which has been freed from Mg^{2+} ions (see Experimental Methods) and subsequently dialyzed to remove the Na^+ ions. The tRNA in such a solution still contains a small amount of bound Mg^{2+} ions which are not removed by our procedure. Addition of Na^+ ions to this solution of tRNA causes an increase in the emission yield, an effect which levels off at a concentration of approximately 0.1 N Na^+. However, a small addition of Mg^{2+} ions to this solution increases again the emission yield significantly. Since no additional stacking takes place we think that what is

Table II

ϕ/τ (s^{-1})	20 °C	−196 °C
4-tU in solution	1200	120
4-tU in tRNA	300	90

being seen is the formation of the above tertiary structure caused by addition of Mg^{2+} ions, a structure which shields the chromophore from the ambient solution. That the vicinity of the 4-tU residue within the tRNA becomes better protected upon addition of Mg^{2+} ions can also be seen from the concurrent increase in the excited-state lifetime which parallels the increase in quantum yield.

That 4-tU in the folded tRNA molecule is well protected from external influences is also seen by the very low quenching constants of oxygen and of Cl^- ions in the solution containing Na^+ and Mg^{2+} ions. Quenching rates by O_2 have lately been employed to study dynamic changes in the tertiary structure of proteins (Lakowicz and Weber, 1973; Saviotti and Galley, 1974). Similarly the low quenching rates in tRNA might be used to study the kinetics of the conformational changes required for the oxygen molecule to reach the excited 4-tU residue in the tRNA interior.

In the temperature dependence of the emission from 4-tU within tRNA in presence of Na^+ and Mg^{2+} ions, we can discern three stages. In the lower temperature range between 10 and 40 °C, the emission yield decreases twice as fast as for free 4-tU in solution. It seems that in this range the (tertiary) structure of tRNA, in the vicinity of the 4-tU residue, undergoes some gradual destruction. In the middle range between 45 and 60 °C practically no change in the emission yield occurs. Here, therefore, the structure seems to be conserved in spite of the increase in temperature. That the structure of tRNA in the vicinity of the 4-tU base is much more resistant to disruptions in the presence of Mg^{2+} than in the presence of Na^+ ions only is indicated by the higher "melting temperature" (see Figure 2) of the molecule. During this high-temperature melting stage, apparently any tertiary structure still left disappears together with the secondary structure.

Within the limitations of our equipment, the above disruption of structures was very similar for both $tRNA^{Val}$ and for a mixture of the various tRNA molecules. A more refined technique might perhaps reveal differences between the various specific tRNA types.

Another indication for structural effects can be seen in the wavelength dependence of the emission of the 4-tU residue. The emission of the free base, 4-tU, and of a salt-free solution of tRNA lies at ~550 nm. Upon addition of Na^+ ions the emission from tRNA shifts gradually toward 530 nm, in 0.1 N Na^+ (see Figure 1c). No further changes in wavelengths are observed when Mg^{2+} ions are added to the solution. It seems, therefore, that this blue shift of emission is due to an effect within the base-paired stack similar to what is measured by the hypochromicity which also is almost unaffected by the presence of Mg^{2+} ions.

That the 4-tU luminescence reflects structural features of tRNA in its vicinity can also be deduced from a comparison between the quantum yield and the decay of 4-tU in solution and in the tRNA molecule. The low temperature emission dependence and the resolution of the spectrum at 77 K (Figure 3) show that in both cases we are dealing with similar triplet emissions. We had previously proposed that the short wavelength emission (λ 475 nm) represents the triplet of 4-tU while the long wavelength emission (λ ~550 nm at room temperature) is a triplet which derives by a fast first-order process either directly from the singlet or from the 4-tU triplet. This first-order process was thought to involve either an intramolecular process or a solvent rearrangement in the neighborhood of the excited chromophore. In view of the rather rigid environment of 4-tU within the tRNA network, the latter process seems somewhat improbable.

The data in Table I show that, though in a rigid medium the quantum yield increases appreciably, the lifetime increases to an even greater degree. While the lifetime indicates the extent to which the 4-tU residue is protected from environmental quenching, it is the quantity ϕ/τ which measures the initial population of the emitting state. If we look at the ϕ/τ values for the long wavelength emission of 4-tU, which represents the above state reached by an intramolecular first-order process, the picture illustrated by Table II emerges.

These values show that the largest initial population of the emitting state is achieved by 4-tU in free solution at room temperature (ϕ/τ = 1200). Embedding the 4-tU residue in the native tRNA network already impedes the above first-order process so that the initial population decreases by a factor of four (ϕ/τ = 300). The even more rigid environment in a low-temperature glass decreases the probability of reaching the (long wavelength) emitting state further till a value of $\phi/\tau \sim 100\ s^{-1}$ is reached. It seems, therefore, that a degree of free molecular movement is necessary for the intramolecular process, which forms the species emitting in the 530–550 nm range, to take place.

In conclusion, we feel that both quantities τ and ϕ/τ might be well suited to yield information about structural changes in the vicinity of the 4-tU residue in tRNA.

References

Barrell, B. G., and Sanger, F. (1969), *FEBS Lett. 3,* 277.

Berlman, I. B. (1965), in Handbook of Fluorescence Spectra, New York, N.Y., Academic Press, p 188.

Calvert, J. G., and Pitts, J. N. (1966), in Photochemistry, New York, N.Y., Wiley, p 799.

Cole, P. E., Yang, S. K., and Crothers, D. M. (1972), *Biochemistry 11,* 4358.

Dourlent, M., Yaniv, M., and Helene, C. (1971), *Eur. J. Biochem. 19,* 108.

Favre, A. (1974), *Photochem. Photobiol. 19,* 15.

Favre, A., Michelson, A. M., and Yaniv, M. (1971), *J. Mol. Biol. 58,* 367.

Favre, A., Reques, B., and Fourrey, J. L. (1972), *FEBS Lett. 24,* 209.

Fresco, J. R., Adams, A., Ascione, R., Henley, D., and Lindahl, T. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31,* 527.

Henley, D. D., Lindahl, T., and Fresco, J. R. (1966), *Proc. Natl. Acad. Sci. U.S.A. 55,* 191.

Hilbers, C. W., Shulman, R. G., and Kim, S. H. (1973), *Biochem. Biophys. Res. Commun. 55,* 953.

Holley, R. W., Apgar, J., Everett, G. A., Madison, J. T., Marguisse, M., Merril, S. H., Penwick, J. R., and Zamir, A. (1965), *Science 147,* 1462.

Kearns, D. R., Lightfoot, D. R., Wong, K. L., Wong, Y. P., Reid, B. R., Cary, L., and Shulman, R. G. (1973), *Ann. N.Y. Acad. Sci. 222,* 324.

Kim, S. H., Quigley, G. J., Suddath, F. L., McPherson, A., Sneden, P., Kim, J. J., Weinzienl, J., and Rich, A. (1973), *Science 179,* 282.

Kim, S. H., Suddath, F. L., Quigley, G. J., McPherson, A., Sussman, J. L., Wang, A. H. J., Seeman, N. C., and Rich,

A. (1974), *Science 185,* 435.
Lakowicz, J. R., and Weber, G. (1973), *Biochemistry 12,* 4171.
Lipmann, F. (1969), *Science 164,* 1024.
Lipsett, M. N. (1965a), *J. Biol. Chem. 240,* 3975.
Lipsett, M. N. (1965b), *Biochem. Biophys. Res. Commun. 20,* 224.
Lipsett, M. N., and Doctor, B. P. (1967), *J. Biol. Chem. 242,* 4072.
Pochon, F., Balny, C., Scheit, K. H., and Michelson, A. M. (1971), *Biochim. Biophys. Acta 228,* 49.
Pochon, F., and Cohen, S. S. (1972), *Biochem. Biophys. Res. Commun. 47,* 720.
Saviotti, M. L., and Galley, W. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4154.
Seno, T., Kobayashi, M., and Nishimura, S. (1969), *Biochim. Biophys. Acta 174,* 71.
Shalitin, N., and Feitelson, J. (1973), *J. Chem. Phys. 59,* 1045.
Shulman, R. G., Hilbers, C. W., Kearns, D. R., Reid, B. R., and Wong, Y. P., (1973b), *J. Mol. Biol. 78,* 57.
Shulman, R. G., Hilbers, C. W., Wong, Y. P., Wong, K. L., Lightfoot, D. R., Reid, B. R., and Kearns, D. R. (1973a), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2042.
Wong, K. L., Bolton, P. H., and Kearns, D. R. (1975), *Biochim. Biophys. Acta 383,* 446.
Wong, K. L., and Kearns, D. R. (1974), *Nature (London) 252,* 738.
Yaniv, M., and Barrell, B. G. (1969), *Nature (London) 222,* 278.
Zachau, H. G. (1969), *Angew. Chem., Int. Ed. Engl. 8,* 711.

Spatial Relationship of the σ Subunit and the Rifampicin Binding Site in RNA Polymerase of *Escherichia coli*†

Cheng-Wen Wu,*,‡ Lynwood R. Yarbrough, Felicia Y.-H. Wu, and Zaharia Hillel

ABSTRACT: σ subunit of *Escherichia coli* RNA polymerase is known to stimulate specific RNA chain initiation. Rifampicin, an inhibitor of RNA chain initiation, binds to a single site on the β subunit of RNA polymerase. We have used the fluorescence energy transfer technique to deduce proximity relationships of σ subunit and the rifampicin binding site on the enzyme. Isolated σ subunit was covalently labeled with fluorescent donors in two ways: specific labeling of a single sulfhydryl residue with *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate (1,5-I-AENS) and nonspecific labeling on the surface of the protein with dansyl chloride (Dns-Cl) adsorbed on Celite. The labeled σ subunits were biologically active and formed a stoichiometric complex with core polymerase. The efficiency of energy transfer was obtained from the fluorescence intensity and the excited-state lifetime of the σ-labeled holoenzyme in the presence and absence of rifampicin, which served as an energy acceptor. The transfer efficiency (2%) from AENS to rifampicin placed AENS somewhere between 42 and 85 Å away from the rifampicin binding site. The rotational mobility of the donor was determined by nanosecond fluorescence depolarization spectroscopy, while the acceptor orientation was assumed to be fixed at some unknown angle. The efficiency measured for energy transfer from Dns to rifampicin was 10% in the presence of 0.2 M KCl. The distance from the surface of σ subunit to the rifampicin binding site was calculated to be 27–38 Å for a model having a randomly distributed and oriented array of donors on the surface of a spherical σ subunit of 31-Å radius. Our results indicate that rifampicin does not inhibit the initiation of transcription by RNA polymerase through a direct interaction with σ subunit. In addition, energy transfer measurements under low salt conditions suggest that in RNA polymerase dimer the two rifampicin binding sites are symmetric with respect to each σ subunit.

The σ subunit of *Escherichia coli* RNA polymerase is known to stimulate specific initiation that yields asymmetric transcripts resembling the in vivo RNA products (Burgess et al., 1969; Travers and Burgess, 1969). Since rifampicin, a specific inhibitor of the initiation of RNA chains, has been shown to bind to a single site on RNA polymerase (Zillig et al., 1970), it is of interest to determine the spatial relationship of the rifampicin binding site and σ subunit in RNA polymerase.

Singlet energy transfer can be used to determine proximity relationships between sites on macromolecules which have been specifically labeled with fluorescent probes (Stryer, 1968). This technique can also be used to estimate intersubunit distances in multisubunit protein complexes by means of random surface labeling (Gennis and Cantor, 1972). This paper reports the use of singlet energy transfer to measure the distances from the rifampicin binding site on RNA polymerase to the surface of σ subunit randomly labeled with dansyl chloride (Dns-Cl[1]) and

† From the Department of Biophysics, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461. *Received August 4, 1975.* This investigation was supported by research grants from the National Institutes of Health (GM-19062) and the American Cancer Society (BC94A).

‡ C.-W. Wu is a Research Career Development awardee of the National Institutes of Health.

[1] Abbreviations used are: 1,5-I-AENS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate; AENS, *N*-(acetylaminoethyl)-5-naphthylamine-1-sulfonate; AENS-σ, fluorescent labeled σ subunit; Dns-Cl, 5-dimethylamino-1-naphthalenesulfonyl chloride or dansyl chloride; Dns, dansyl group; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.