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Time-Resolved Absorption, Circular Dichroism, and Emission of tRNA. Evidence That the Photo-Cross-Linking of 4-Thiouridine in tRNA Occurs from the Triplet State†

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ABSTRACT: The time-resolved optical density (TROD) and time-resolved circular dichroism (TRCD) spectra of the lowest triplet state of 4-thiouridine (4t-Urd) in aqueous solutions of tRNA are reported. The TROD spectrum is consistent with the triplet state being primarily in the thione tautomer. The intersystem crossing yield to the triplet is 0.35 and 0.27 (±10%), respectively, with and without 10^{-2} Mg^{2+} added to the solution. Upon addition of increasing amounts of I^- to solutions of tRNA, the initial triplet yield decreases, the rate of the observed triplet decay increases, and the quantum yield of internal photo-cross-linking decreases for the 4t-Urd chromophore. The results show quantitatively that the near-UV-induced photo-cross-linking reaction in tRNA occurs from the triplet state of 4t-Urd. From the TRCD spectrum the dissymmetry factor ($\Delta\epsilon/\epsilon$) of some of the triplet–triplet absorption bands is shown to be significantly larger than for any of the ground-state absorption bands. Two CD transitions are seen in the triplet–triplet spectrum which are obscured in the TROD spectrum by the strong ground-state bleaching signal near 335 nm. This shows that TRCD may be useful, in some cases, in locating electronic transitions that are not observed in TROD spectra.

Transfer ribonucleic acid (tRNA)[1] has been shown to be the photoactive species in the near-UV inhibition of the growth of *Escherichia coli*. The chromophore responsible for absorption of this light is the somewhat rare base 4-thiouridine (4t-Urd) (Favre & Thomas, 1981; Jagger, 1975; Peak et al., 1983; Caldeira de Araujo & Favre, 1985). For tRNA in solution, 4t-Urd has been shown to photo-cross-link with a

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[1] Abbreviations: CD, circular dichroism; CPL, circularly polarized luminescence; Cyt, cytidine; DMTU, 1,3-dimethyl-4-thiouracil; OMA, optical multichannel analyzer; PMMA, poly(methyl methacrylate); tRNA, transfer ribonucleic acid; TRCD, time-resolved circular dichroism; TROD, time-resolved optical density; 4t-Urd, 4-thiouridine.

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cytidine, and the quantum yield has been shown to increase when Mg^{2+} is added to the solution ($\phi = 5 \times 10^{-3}$, with added Mg^{2+}) (Buckingham et al., 1973; Favre et al., 1971, 1972; Yaniv et al., 1971). When free in aqueous solution, 4t-Urd emits from its triplet state with a lifetime of 250 ns at 295 K (Favre, 1974). When this chromophore is in tRNA dissolved in an aqueous solution with added Mg^{2+}, its triplet emission lifetime at 295 K is reported to be 6.6 μs (Shalitin & Feitelson, 1976).

We previously reported the excited-state triplet–triplet absorption spectrum of 4t-Urd in solution and showed that it is consistent with the triplet 4t-Urd being primarily a thione tautomer (Milder & Kliger, 1985). 4t-Urd in tRNA is amenable to spectroscopic study, as its lowest strong absorption (λ_{max} = 334 nm) is significantly to the red of the absorption of the other nucleic acid bases. In the present work the triplet–triplet absorption spectrum of 4t-Urd in solution is compared with the analogous spectrum in tRNA. Also, the time-resolved circular dichroism (TRCD) spectrum is reported for the triplet–triplet absorption of 4t-Urd in tRNA. These and other results show that the excited state of 4t-Urd in tRNA is primarily in the thione tautomer and that the decay of the triplet is primarily to the ground state. The circular dichroism spectrum of the triplet state shows the existence of at least two absorption bands predicted to exist in the triplet–triplet spectrum by INDO/S calculations that are not directly observed in the unpolarized spectrum.

We also present the results of the quenching of the triplet state of 4t-Urd in tRNA by iodide. The effect of iodide on the initial triplet production yield, the triplet decay kinetics, and the emission and photoproduct yields are shown to be intimately connected. While previous work suggested that the near-UV-induced cross-linking reaction occurs from the lowest triplet state (Favre, 1974), the current results are much stronger evidence that this reaction must indeed emanate from this triplet.

Materials and Methods

Time-resolved optical density (TROD) measurements were taken on an apparatus that has been described in detail previously (Lewis et al., 1987; Milder & Kliger, 1985). Briefly, the sample was excited with a 7-ns pulse at 355 nm from the third harmonic of a Nd:YAG laser (Quanta Ray, DCR-1). The probe beam was produced by a xenon flashlamp (τ = 5 μs) which was monitored to optimally yield either spectral or kinetic information. In the spectral mode the probe beam was focused into a Jarrell-Ash Monospec 27 polychromator and was dispersed across a PAR 1420 optical multichannel analyzer (OMA) gated on for 10 ns by a PAR 1302 pulser. In the kinetic mode the probe beam was focused into a Pacific Precision Instruments 0.45-m monochromator and wavelength selected. The resulting light was detected with a fast-rise (τ < 2 ns) photomultiplier whose output was digitized and averaged by a Tektronix 7912AD/4041.

Time-resolved circular dichroism (TRCD) spectra were obtained by using the detection scheme described above except that the probe beam optics were changed, in a manner previously described, to yield CD information (Kliger & Lewis, 1987; Lewis et al., 1985; Milder et al., 1988). Since the probe light levels are much lower in the TRCD experiments, the OMA was gated with a PAR 1304 pulser with a 1.0-μs gate.

Ground-state CD spectra were taken on an Aviv 60AD spectropolarimeter, and UV–vis spectra were taken on an IBM 9420 spectrophotometer. Emission spectra were taken on a Spex Fluorolog spectrofluorometer, and emission lifetimes were obtained by using the TROD apparatus in the kinetic mode. Kinetic lifetimes were determined by obtaining the best visual fit of the data to either a mono- or biexponenetial decay curve.

Samples of *E. coli* tRNA B from different lots were obtained from Schwarz-Mann and were used as received. 4t-Urd was obtained from Sigma, and 1,3-dimethyl-4-thiouracil (DMTU) was made as previously described (Elion & Hitchings, 1947; Milder & Kliger, 1985). Poly(methyl methacrylate) (PMMA) films with dissolved DMTU were made by dissolving the chromophore in CH_2Cl_2 and mixing this solution with low molecular weight beads of PMMA (obtained from Aldrich). The resultant viscous solution was placed on a microscope slide, and the solvent was allowed to slowly evaporate. This provided transparent films with a minimum number of cracks.

Solutions of tRNA and 4t-Urd were made in doubly distilled water. In most tRNA solutions $MgCl_2$ was added to give a solution that was 10^{-2} M in Mg^{2+}. tRNA was added to the solutions to give an absorbance in a 1-cm path of 0.4 at the absorption maximum at 334 nm. On the basis of the reported extinction coefficient of unfractionated tRNA of 7300 M^{-1} cm^{-1} (Willick & Kay, 1971), the concentration of tRNA in our solutions is calculated to be 5×10^{-5} M. The pH of the solutions were approximately 5.3, and the pH did not change upon photolysis of the solute tRNA. It was necessary to test if the absence of buffer in our solvent influenced the state of the tRNA (Cole et al., 1972). For the 10^{-2} M Mg^{2+} solution, we observed that the CD (Willick & Kay, 1971) and the percent yield of cross-linked 4t-Urd/Cyt as a percentage of the photoproduct (Debreuil et al., 1986) were consistent with the tRNA being in the native form I. It is probable that the tRNA in distilled water without added Mg^{2+} is not in the native form I (Debreuil et al., 1986).

As 4t-Urd is photochemically active both free in solution and in tRNA, it was necessary to flow the solutions through the actinic region during the transient absorption experiments to ensure excitation of intact 4t-Urd. As the triplet is quenched by O_2, solutions of free 4t-Urd in solution were deoxygenated by bubbling them with N_2 for at least 15 min. Similar deoxygenation was not done for solutions of tRNA, as 4t-Urd is not quenched by O_2 when it is incorporated in this species (Shalitin & Feitelson, 1976).

Results and Discussion

Triplet Lifetimes. Previous work has shown that 4t-Urd in fluid solution at 295 K has an intersystem crossing yield to the lowest triplet of 1.0 ± 0.1 and a lifetime of between 15 and 320 ns, depending on the solvent (Milder & Kliger, 1985). When the chromophore is at 77 K in a rigid glass, the emission decay is biexponential with lifetimes of 50 and 100 μs (Shalitin & Feitelson, 1973). Previous reports have shown that the emission lifetime of the triplet state of 4t-Urd in purified $tRNA^{Val}$ in aqueous solution with added Mg^{2+} at 293 K is 6.6 μs (Shalitin & Feitelson, 1976). We measured the decay time of the triplet of 4t-Urd in the mixed tRNAs used in the current study by following the transient absorption of the triplet state of 4t-Urd at 293 K in aqueous solution with and without 10^{-2} M Mg^{2+} added. The absorption at various times following excitation of tRNA in 10^{-2} M Mg^{2+}(aq) is shown on Figure 1. The transient absorption and the transient bleaching signals decayed simultaneously at all wavelengths between 310 and 600 nm. The lifetimes we obtain using a single-exponential fit are 6.1 and 3.2 μs for solutions with and without added Mg^{2+}, respectively. These values are crude due to the small number of time points, so we obtained the decay of the emission using the kinetic mode of observation. For the case of tRNA in 10^{-2} M Mg^{2+}(aq), a biphasic emission decay was

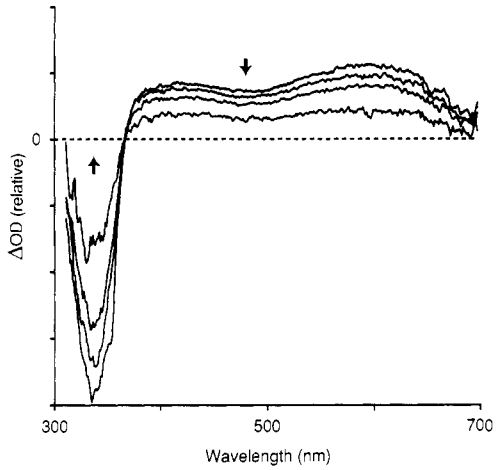

FIGURE 1: Transient absorption spectrum of 4t-Urd in tRNA (5×10^{-5} M) dissolved in water with 10^{-2} M added Mg^{2+} (pH = 5.3). The sample was excited at 355 nm with a 7-ns pulse, and the spectra were taken with a 10-ns gate beginning 50 ns, 250 ns, 1 μs, and 4 μs after excitation. At all wavelengths, the magnitude of the signal decreases monotonically with time after excitation. Each spectrum represents 64 averages.

observed. The lifetimes of the two components of the decay are 2.4 and 8.3 μs, with the fast component representing 30% of the decay. The preparation of tRNA used was a mixture of tRNAs. The biphasic emission decay suggests that there may be differences in the triplet decay rates for 4t-Urd in the different tRNAs in our samples. As the decay of the TROD spectrum is, within experimental error, the same at all wavelengths, the excited-state spectra of the constituent tRNAs are similar.

To test whether rigidity is a factor in increasing the lifetime of 4t-Urd in tRNA relative to fluid solution, rigid solutions of 1,3-dimethyl-4-thiouracil (DMTU) in PMMA were studied at room temperature. DMTU has been shown to have triplet-state properties that are very similar to those of 4t-Urd (Milder & Kliger, 1985). Its triplet decays with a lifetime of less that 250 ns in fluid solution at 295 K. In PMMA, DMTU had a much longer lived emission which decays biexponentially with lifetimes that depend on the history of the glass. Typical lifetimes obtained were in the range 5–15 and 1–3 μs, with the shorter lived emission representing about 60% of the decay. That there is a biphasic decay which changes with the history of the sample implies that there is more than one environment for the DMTU in PMMA. It thus appears that the rigidity of the surrounding medium lengthens the triplet lifetime and is an important factor corresponding to the longer triplet lifetime of 4t-Urd in tRNA. The lifetime is equal to the inverse of the sum of the radiative and nonradiative rate constants of the triplet, $\tau_0 = 1/(k_r + k_{nr})$. As the emission yield is small, $k_{nr} \gg k_r$, and the lifetime is essentially the inverse of the nonradiative rate constant. Thus, environmental rigidity contributes to the decrease of the nonradiative rate constant of the 4t-Urd triplet state in tRNA.

Transient Absorption and Triplet Yield. In our previous study of 4t-Urd in solution we showed that the triplet–triplet spectrum is consistent with the excited-state being in the thione tautomer (Milder & Kliger, 1985). In the present study, absorption spectra of the triplet state were taken 50 ns after photolysis for 4t-Urd in aqueous solution as well as in tRNA in aqueous solutions with and without added Mg^{2+}. These triplet–triplet spectra are shown in Figure 2. The spectrum of the free 4t-Urd is corrected for the partial decay of its excited state at the detection time. Figure 2a shows a comparison of the transient absorption and bleaching signals when

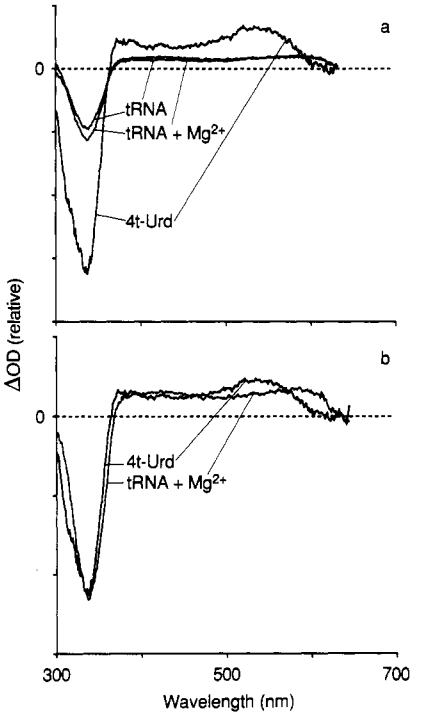

FIGURE 2: (a) Transient difference spectrum of 4t-Urd in water and in tRNA in aqueous solution with and without added Mg^{2+}. Each sample was excited at 355 nm, and each solution had an absorption of 0.3 at 355 nm. The spectra were obtained 50 ns after the laser pulse and represent 64 averages. The spectra have been corrected for partial decay of the excited state at the detection time. (b) Comparison of the transient difference spectrum of 4t-Urd in water and in tRNA in water with added Mg^{2+}. The spectra have been adjusted so that the minima of the transient bleaching are the same magnitude.

each sample is probed under identical photolysis conditions (e.g., same optical density at 355 nm, same laser power). As the extinction coefficients at 355 nm are somewhat different for 4t-Urd in solution and in tRNA in solution, we took care to avoid saturating the absorption of the chromophore, as this would lead to excitation of different amounts of 4t-Urd in the two cases. It is clear that under identical excitation conditions 4t-Urd has a much larger transient bleaching in solution than it does in tRNA in solution. Previous work showed that for 4t-Urd in solution the triplet-state absorption is probably not large through the ground-state bleaching band centered at 335 nm. Assuming that in all three spectra the absorption of the triplet state is small and equivalent in magnitude near the maximum of the bleaching, the relative triplet yield is proportional to the bleaching signal. Using the previously determined intersystem crossing yield of 1.0 ± 0.1 for 4t-Urd in solution, it is possible to estimate (to within about 10%) the intersystem crossing yield of 4t-Urd in tRNA to be 0.35 and 0.27 when Mg^{2+} is and is not added to the solution, respectively.

The decrease in intersystem crossing for 4t-Urd when it is in tRNA must come from either increased internal conversion from the excited singlet state to the ground state or photoproduct production. Since no emission is seen from the singlet state, one can make a conservative estimate of the upper limit of the quantum yield of fluorescence (ϕ_f) to be 10^{-4}. The lowest singlet is an $n\pi^*$ state and probably has an extinction coefficient of on the order of 500 M^{-1} cm^{-1} (Igarashi-Yamamoto et al., 1981; Milder & Kliger, 1985). Taking the crude estimate of the natural lifetime of a state as $\tau_N = 10^{-4}/\epsilon$ (Calvert & Pitts, 1966), this gives the upper limit of the actual lifetime ($\tau_f = \tau_N \phi_f$) of the singlet $n\pi^*$ state as about 2×10^{-11}

s. Thus, the internal conversion and/or the photoreactivity from this state must be rapid in tRNA to compete with the intersystem crossing that takes place on this time scale. It is not obvious how the rigidity of the polymer would directly influence the rate of internal conversion or intersystem crossing. However, for free 4t-Urd the rigidity of the solution has been shown to have a large influence on many excited-state properties (Shalitin & Feitelson, 1976).

For the emission decay of the triplet in free 4t-Urd, the value of ϕ^0/τ^0 (ϕ^0 = observed triplet emission quantum yield, τ^0 = observed emission lifetime) decreases by a factor of 10 upon going from 20 °C fluid solution to −196 °C rigid glass. ϕ^0/τ^0 equals $k_r\phi_{isc}$, where k_r is the unquenched (natural) radiative rate of the emissive triplet state and ϕ_{isc} is the initial yield of the emissive triplet state. The value of k_r should not vary significantly, as it essentially depends on the intensity of the absorption between the ground and the emitting states (Strickler & Berg, 1962). It has been shown that for 4t-Urd the emitting state is the same $^3\pi\pi^*$ state both in room temperature fluid solution and in a low-temperature rigid glass (Milder & Kliger, 1985). There is no obvious reason why the transition intensity to such a state would depend strongly on the solvent rigidity. Thus, it seems that the intersystem crossing yield must be affected by rigidity. ϕ_{isc} equals $k_{isc}/(k_{isc} + k_{other})$, where k_{other} represents the rate of decay from other processes such as internal conversion and chemical reaction. In the present case, it is possible that ϕ_{isc} decreases due to the rigidity inhibiting the vibrations in the chromophore that lead to the coupling between the lowest excited singlet state and the triplet manifold. Previous work has shown that out of plane distortions can be important in coupling which leads to enhanced nonradiative decay rates for a number of heterocyclic chromophores (Lim, 1986). Another possible explanation for the decreased intersystem crossing of 4t-Urd when it is in tRNA may be that the lowest excited singlet state ($^1n\pi^*$) is chemically quenched by a nucleic acid base to which it is in close proximity. In tRNA the cytidine at position 13 (Cyt_{13}) is in close proximity to the 4t-Urd at position 8 (Rich & RajBhandary, 1978), and it might quench the singlet. The quenching may be by electron transfer from either the lone pair on the nitrogen or the conjugated C_4–C_5 double bond to the hole on sulfur in the $n\pi^*$ state. Since the putative ions produced cannot diffuse away, the back electron transfer would probably occur in picoseconds (Fox et al., 1987; Mataga et al., 1986), leading to rapid production of the starting bases. In this view, changes in the structure caused by binding of Mg^{2+} may affect the relative position of 4t-Urd and its quencher. This could decrease the rate of quenching and allow for a greater yield of intersystem crossing. However, if the intersystem crossing yield is primarily affected by a change in the intersystem crossing rate due to rigidity, then it may be that the structural changes that accompany the binding of Mg^{2+} change the rigidity of the microenvironment of the 4t-Urd.

Figure 2b shows a comparison of the TROD spectra of 4t-Urd in solution and in tRNA when their bleaching signals are scaled to give the same value at the minimum. The triplet–triplet spectra of both are broad and have very nearly the same intensity between 370 and 600 nm. This suggests that they emanate from the same state, which for 4t-Urd has been shown to be the $\pi\pi^*$ triplet of the thione tautomer (Milder & Kliger, 1985). The only obvious difference in these spectra is that the peak at 540 nm for 4t-Urd in solution appears to shift to 580 nm in tRNA. This shift does not imply there is a difference in the absorbing state, as this band has been observed in DMTU to lie at 590 nm (Milder & Kliger, 1985), and the triplet of this species must be the $\pi\pi^*$ of the thione tautomer.

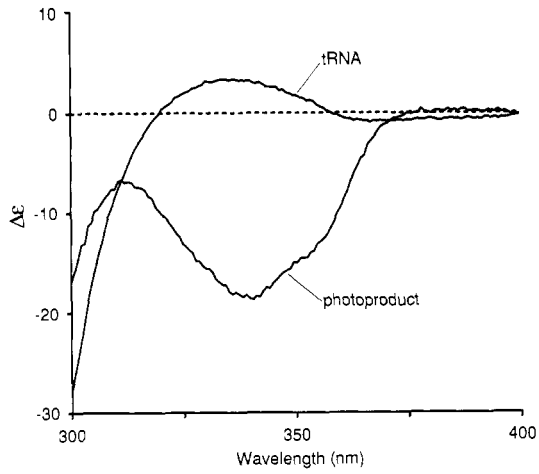

FIGURE 3: Circular dichroism spectrum of 4t-Urd in tRNA in aqueous solution with added Mg^{2+} and the CD obtained for the same solution after exhaustive photolysis at 355 nm. Photolysis gives a solution with a very similar near-UV absorption but a loss of transient absorption and bleaching after photolysis.

An interesting question is why the triplet lifetime of 4t-Urd increases when it is in tRNA versus free in solution while its initial yield decreases. If the S_1 state is effectively quenched by the Cyt_{13}, it would seem at first glance that the T_1 state should also be quenched, leading to a shortening of its lifetime. A previous study showed that the triplet of 4t-Urd in solution is quenched at near the diffusion-controlled rate by tertiary amines in solution (Milder & Kliger, 1985). However, primary amines are much poorer electron donors than tertiary amines (Guttenplan & Cohen, 1972), and Cyt_{13} apparently does not quench the triplet. The $^1n\pi^*$ (S_1) state is predicted to be approximately 5000 cm^{-1} (0.6 eV) higher in energy than the $^3\pi\pi^*$ (T_1) (Milder & Kliger, 1985). The S_1 state should thus be about 0.6 V easier to reduce than the T_1 state, and it may be that the S_1 is exclusively quenched. Alternatively, it may be that rigidity has the same inhibitory affect on the coupling necessary for the intersystem crossing from both the $^1n\pi^*$ and $^3\pi\pi^*$ states. Thus, the rate of intersystem crossing would be lower for both states, leading to a lowering of the singlet-to-triplet yield and a lengthening of the triplet lifetime.

Circular Dichroism of Ground-State 4t-Urd and the Photoproduct. The circular dichroism spectrum (CD) of 4t-Urd in solution and in tRNA has been previously reported (Willick & Kay, 1971). The magnitude of the CD of the chromophore is much larger when it is in tRNA, and it is enhanced in tRNA when Mg^{2+} is added to its solutions. This enhanced CD is probably due to the coupling of the electric dipole transitions between 4t-Urd and other nearby nucleic acid bases. Mg^{2+} binding effects the tertiary structure of the tRNA and changes the relative positions of 4t-Urd and the bases with which it couples.

Upon photolysis at 355 nm, the near-UV absorption spectrum of a solution of tRNA does not change much, consistent with form I tRNA photobehavior (Favre, 1971; Dubreuil et al., 1986). However, we observe that the magnitude of the transient absorption decreases with increasing photolysis and that concomitantly the CD spectrum changes. The photoproduct does not have any apparent transient absorption or bleaching on the nanosecond time scale when it is excited at 355 nm. Figure 3 shows the near-UV CD spectrum of tRNA and the product of exhaustive photolysis. Though their near-UV absorption is known to be very similar, it is obvious

that their CD is very different. In fact, we have found that the photoproduct is due primarily to cross-linking between the 4t-Urd and the cytidine in position 13, as the product can be reduced with $NaBH_4$ to give a species that absorbs at 390 nm (Ofengand & Bierbaum, 1973; Dubreuil et al., 1986).

Emission Quenching and Photoproduct Formation. Previous work has reported that upon chemical quenching of the triplet emission of 4t-Urd in tRNA there is a concomitant decrease in the quantum yield of photoproduct formation (Favre, 1974). This was taken to show that the photoproduct emanated from the triplet state. The bimolecular rate constants for quenching of the emission of the 4t-Urd triplet in tRNA are low (Shalitin & Feitelson, 1976; Favre, 1974), and high concentrations of quenchers were necessary in these studies. It is thus possible that the quencher actually acted to quench the singlet. Similar to the expected relative rates of internal quenching discussed above, the singlet should be quenched at a bimolecular rate that is much higher than that for the triplet. Thus, even though the singlet lifetime is very short ($<2 \times 10^{-11}$ s), it could be the state which is quenched. Quenching of the singlet could also explain the concomitant quenching of the photoproduct formation and the triplet emission. If the singlet is quenched and the photoproduct emanates from the triplet, then the decreased yields are both due to a decreased yield of intersystem crossing. However, if the photoproduct emanates from the singlet, then the decreased photoproduct yield is due directly to the quenching of the singlet. In this case, the intersystem crossing to the triplet will be quenched concurrently, leading to the concomitant loss of triplet emission.

A previous analysis of the quenching of the triplet emission of 4t-Urd in tRNA by Cl^- reported the bimolecular rate as 2×10^5 M^{-1} s^{-1} (Shalitin & Feitelson, 1976). The current work used I^- as the quencher, as it is a better electron donor and it usually acts as a more efficient quencher. Four experimental parameters were followed upon addition of various amounts of I^- to solutions of tRNA with 10^{-2} M Mg^{2+}. First was the quenching of the triplet emission as observed in a conventional fluorometer. Second was the quenching of the two components of the triplet emission decay observed at 580 nm. Third was the relative apparent intersystem crossing yield, determined by the relative size of the maximum of the transient bleaching of the ground state in the transient absorption spectrum directly (<10 ns) after excitation. This was done by observing the relative transient bleaching signal size at 334 nm by use of the kinetic mode of observation on a freshly prepared sample. Fourth was the relative rate of photoproduct formation. This was determined by observing the relative percentage of the loss of the initial transient bleaching at 335 nm after a specified number of laser pulses were absorbed by the sample.

The conventional quenching experiment gave a Stern–Volmer plot of ϕ^0_{em}/ϕ_{em} versus $[I^-]$ that curved upward in the region of highest iodide concentration (Figure 4). The low iodide concentration pseudolinear region of the plot gives a slope of approximately 6 M^{-1}. Since simple dynamic quenching should lead to a linear Stern–Volmer plot, $\phi^0/\phi = 1 + k_q\tau^0[Q]$ (k_q = bimolecular quenching rate, τ^0 = unquenched lifetime, [Q] = quencher concentration], another quenching process must also be occurring. We tested this by looking at the decrease in the lifetime of the two components of the triplet decay and the decrease in the initial triplet yield with increasing iodide concentration. A linear Stern–Volmer plot of τ^0/τ versus $[I^-]$ was obtained for both the fast and slow component of the emission decay. The slopes (the Stern–Volmer constant, $K^3_{sv} = k_q\tau^0$) are 7.3 and 5.6 M^{-1}, respectively. This gives the iodide quenching rate for the fast and slow components as 3.2×10^6 and 1.5×10^6 M^{-1} s^{-1}, respectively. It is also apparent upon increasing the concentration of iodide that the initial yield of the triplet (ϕ_t) decreases slightly. The Stern–Volmer plot of ϕ^0_t/ϕ_t versus $[I^-]$ was found to be linear, and a Stern–Volmer constant (K^1_{sv}) of 0.54 M^{-1} was obtained.

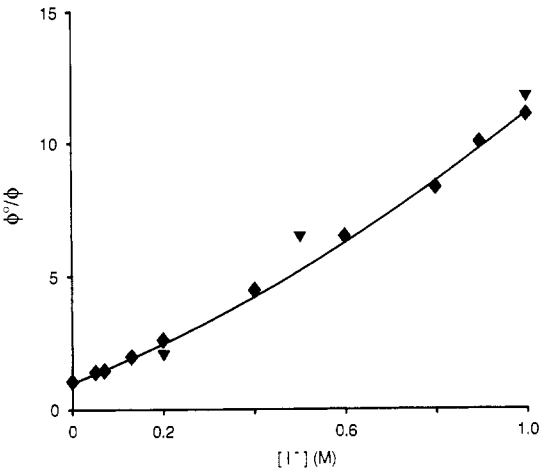

FIGURE 4: Stern–Volmer plot of the quenching, upon addition of I^- as quencher, of the yield of triplet emission (♦) and the yield of photoproduct formation (▼) of 4t-Urd in tRNA dissolved in water with added Mg^{2+}. The triplet emission was obtained on a conventional fluorometer with excitation at 334 nm. The relative photoproduct yield was determined by observing the rate of decrease of the initial transient ground-state bleaching at 335 nm upon excitation at 355 nm. The line represents $\phi^0/\phi = 1 + (K^1_{sv} + K^3_{sv})[I^-] + K^1_{sv}K^3_{sv}[I^-]^2$, with $K^1_{sv} = 0.54$ M^{-1} and $K^3_{sv} = 6.15$ M^{-1}, the Stern–Volmer constants for intersystems crossing and triplet emission lifetime quenching (see text).

The decrease in the apparent initial yield of triplet upon increasing iodide concentration could be due to either of two factors. One is the quenching of the singlet state by iodide, either by dynamic or static quenching. The other is the static quenching of the triplet state. Either process can give a linear Stern–Volmer plot, $\phi^0_t/\phi_t = 1 + K^1_{sv}[I^-]$, where ϕ_t is the initial triplet signal size. Regardless of the mechanism, the decrease in the initial triplet yield upon addition of iodide rationalizes the upward curvature in the Stern–Volmer plot of the triplet emission yield. The Stern–Volmer plot for this case will have the form $\phi^0/\phi = 1 + (K^1_{sv} + K^3_{sv})[I^-] + K^1_{sv}K^3_{sv}[I^-]^2$. A fit of this to the data is shown on Figure 4, with K^1_{sv} as the Stern–Volmer constant obtained for the triplet production quenching and K^3_{sv} as the weighted Stern–Volmer constant (6.15 M^{-1}) from the two triplet decay components.

The relative rate of photoproduct formation upon iodide addition was also determined. This was done by looking at the relative change in the initial ($\tau \simeq 10$ ns) triplet bleaching signal at 334 nm after the same number of laser pulses were absorbed by each sample. ϕ_{pp} represents the percent change in this signal at each iodide concentration, and the ϕ^0_{pp}/ϕ_{pp} values obtained are plotted on Figure 4. We estimate each point is accurate to within about 10%. Within experimental error, these points lie on the line of the triplet emission loss. If the singlet was the precursor state, then the concomitant loss of initial triplet and photoproduct would have been observed. These results clearly indicate that the triplet state is the precursor of the photoproduct.

We attempted to observe the absorption of chemical intermediates that might precede the production of the final photoproduct. At times when all of the triplet had decayed (e.g., 50–200 μs after the laser pulse), no transient absorption

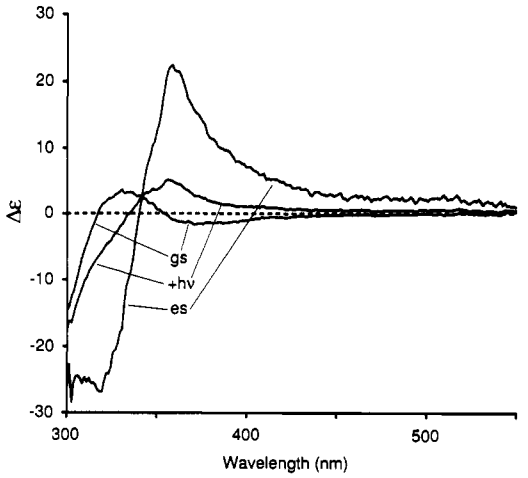

FIGURE 5: TRCD spectra of 4t-Urd in tRNA in aqueous solutions (5×10^{-5} M) with 10^{-2} M added Mg^{2+}. Spectra are the CD of the unexcited ground state (gs) and of the transient obtained in the time window 200–1200 ns after excitation at 355 nm ($+h\nu$). They each represent 1000 averages. Also shown is the CD spectrum of the triplet–triplet absorption (es) which was determined from the unexcited and transient spectra. For this spectrum, 25% of the sample was taken to be converted to the triplet state. This value was determined from the unpolarized transient bleaching signal obtained under identical photolysis conditions with the assumption that the magnitude of the transient absorption is not large compared to the magnitude of the transient bleaching.

between 350 and 700 nm was observed. Since the quantum yield for photoproduct formation is only 5×10^{-3} (Favre et al., 1971; Yaniv et al., 1971), it is possible that such intermediates occur but that due to their low concentrations their absorption is too weak to observe. This does show that the triplet state does not decay in high yield to a long-lived (>50 μs) chemical intermediate which then partitions to either the initial ground-state reactants or the final photoproduct.

Excited-State Circular Dichroism. Figure 5 shows the CD of the ground and lowest triplet states of 4t-Urd in tRNA in 10^{-2} M Mg^{2+}(aq) obtained by using the TRCD apparatus. The ground-state spectrum is very similar to the CD we obtained using a conventional apparatus. In the 1 μs starting 200 ns after excitation of the tRNA at 355 nm, a distinctly different CD signal is observed. In this case, the CD signal is due to the sum of the 4t-Urd triplet states and the remaining 4t-Urd ground states. Thus, to obtain the true CD spectrum of the triplet state, it is necessary to estimate the percentage of the chromophore that is in the excited state and adjust the observed TRCD spectrum. This was done by looking at the size of the unpolarized transient bleaching signal, at the maximum of the bleaching, under identical excitation conditions. It was assumed that the transient triplet absorption at this wavelength is much weaker than the transient ground-state bleaching, and so the signal was analyzed as if it is due only to loss of the ground-state absorption. This will always lead to an underestimation of the triplet yield. However, any discrepancy should be small. Production of the excited-state spectrum assuming an excited-state absorption 5 time weaker than the excited-state bleaching at 334 nm does not alter the basic features of the spectrum. MO calculations have predicted that in this spectral region the absroption will be about a factor of 5 times weaker in the excited state than it is in the ground state (Milder & Kliger, 1985).

Figure 5 shows the CD spectrum of the triplet state calculated from the TRCD spectrum. Its unstructured positive CD from 400 to 550 nm is consistent with the weak absorptions in this region. There is a somewhat stronger positive CD

Table I: Calculated Absorption Spectrum of the Lowest Triplet State of 1-Methyl-4-thiouracil Using INDO/S

triplet state[a]		10^3E (cm^{-1}) (f)[b]	triplet state[a]		10^3E (cm^{-1}) (f)[b]
T_2	$n\pi^*$	3.7 (0.000)	T_{11}	$\pi\pi^*$	30.1 (0.079)
T_3	$\pi\pi^*$	9.5 (0.019)	T_{12}	$n\pi^*$	30.3 (0.035)
T_4	$\pi\pi^*$	16.7 (0.030)	T_{13}	$n\pi^*$	32.3 (0.055)
T_5	$n\pi^*$	18.4 (0.000)	T_{14}	$\pi\sigma^*$	35.4 (0.009)
T_6	$n\sigma^*$	22.4 (0.022)	T_{15}	$\pi\pi^*$	37.8 (0.163)
T_7	$\pi\pi^*$	22.7 (0.049)	T_{16}	$n\pi^*$	38.1 (0.000)
T_8	$\pi\sigma^*$	23.6 (0.009)	T_{17}	$n\sigma^*$	39.2 (0.003)
T_9	$n\pi^*$	27.1 (0.001)	T_{18}	$n\pi^*$	39.4 (0.014)
T_{10}	$\pi\sigma^*$	28.5 (0.012)	T_{19}	$n\sigma^*$	41.3 (0.008)

[a] The orbital designation of the triplet state, T_n, as a single excitation from S_0. [b] Transition energies from T_1, f = oscillator strength. [For details, see Milder and Kliger (1985).]

centered near 360 nm and a negative CD from 300 to 340 nm. These two CDs are particularly interesting as there are no obvious transient absorptions in this region. This can be rationalized by postulating that there are transitions in the triplet–triplet absorption which correlate with these CDs but that they are much weaker than the ground-state absorption. Thus, in the unpolarized spectrum they are obscured by the ground-state bleaching signal. A summary of previously reported INDO/S MO calculations (Milder & Kliger, 1985) is shown on Table I. These calculations predict the existence of three absorptions in the triplet–triplet spectrum between 300 and 330 nm with significant absorption strengths ($T_1 \rightarrow T_{11}$, T_{12}, and T_{13}). It appears that some of these transitions have significant CDs.

The ratio of the CD magnitude to the OD magnitude, the dissymmetry factor ($g_{abs} = \Delta\epsilon/\epsilon$), is greater for the triplet–triplet absorption than for the ground-state absorption. As an example, for 4t-Urd in tRNA the extinction coefficient at 335 nm in the ground-state absorption is about six times larger than it is near 450 nm in the triplet absorption, but the magnitude of the CD for these two absorptions is about the same. Thus, the transitions in the excited-state absorption appear to couple more strongly with the transitions of the neighboring nucleic acid bases than do the ground-state transitions. This result is consistent with previous work on the circularly polarized luminescence (CPL) of 4t-Urd in tRNA (Steinberg et al., 1982). The dissymmetry factor observed in the CPL spectrum ($g_{em} = 2\Delta I/I$) is much greater than the dissymmetry factor seen in the ground-state CD. Theoretical work shows that the dissymmetry factor should be the same for an absorption and emission between the same states if there is no difference in their relaxed structure (Richardson & Riehl, 1977). However, the emission is due to a different transition ($^3\pi\pi^*$) than that to which the ground-state near-UV CD is attributed ($^1\pi\pi^*$). CD and CPL of transitions of 4t-Urd in tRNA probably gain most of their intensity through exciton coupling. The greater dissymmetry in the CPL may be due to a geometry change in the excited state that enhances this coupling. Alternatively, it may be that some transitions from the $^3\pi\pi^*$ state can electronically couple more efficiently with transitions in nearby moieties. Further work on the excited-state absorptions of other molecules with exciton coupled states will be necessary to show if it is common that excited-state transistions couple more efficiently with transitions of neighboring molecules.

Conclusions

The TROD spectrum of 4t-Urd in tRNA shows that the triplet state must be primarily the lowest triplet state of the thione tautomer ($^3\pi\pi^*$). The intersystem crossing yield for

4t-Urd in tRNA in an aqueous solution is 0.35 with added Mg^{2+} and 0.27 without added Mg^{2+}. Since the intersystem crossing yield for 4t-Urd in solution is 1.0, it appears that for the lowest singlet state of 4t-Urd in tRNA either the rate of intersystem crossing is decreased or the rate of transient product formation is accelerated relative to the rates for the chromophore free in solution. For the mixed tRNAs used here, a biphasic decay of the triplet emission is observed in an aqueous 10^{-2} M Mg^{2+} solution. The lifetimes obtained are 2.4 and 8.3 μs, with 30% of the decay being the fast component. Iodide quenches the emission of 4t-Urd in tRNA. The lifetimes of both components of the biphasic triplet decay decrease. For iodide quenching the fast and slow components have Stern–Volmer constants of 7.3 and 5.6 M^{-1}, which give bimolecular quenching rate constants of 3.2×10^6 and 1.5×10^6 M^{-1} s^{-1}, respectively. There is also a decrease in the initial triplet yield. Its loss also follows Stern–Volmer kinetics, with a Stern–Volmer constant of 0.54 M^{-1}. There is a concomitant loss of the yield of triplet emission and photoproduct formation. These results are clear evidence that the photo-cross-linking between the 4t-Urd in the 8-position and the Cyt in the 13-position occurs from the lowest triplet ($^3\pi\pi^*$). The TRCD spectrum obtained of the triplet–triplet absorption shows the existence of two transitions obscured in the TROD spectrum of this state due to the intense ground-state bleaching. This points out the possible utility of TRCD, in some cases, in locating electronic transitions that are not observed in TROD spectra of transient states.

Registry No. 4-t-Urd, 13957-31-8; DMTU, 49785-67-3; Mg, 7439-95-4; iodide, 20461-54-5.

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