

Supplemental Material for:

Huggins, Shapkina and Wollenzien, 2007, Conformational energy and structure in canonical and non-canonical forms of tRNA determined by temperature analysis of the rate of s⁴U8-C13 photocrosslinking

DESCRIPTION OF THE EXPERIMENTS

Identification of the s⁴U8-C13 crosslink

In a previous study involving a UVB irradiation-induced crosslink in yeast tRNA^{Phe} between C48 and U59, a product with a slower mobility than the native mobility was seen (Behlen et al. 1991).

Therefore it was surprising that UVA irradiation of *E. coli* tRNAs would produced a faster migrating product (in Band 2) compared to native tRNA (in Band 1) on the 8% denaturing polyacrylamide gel.

To verify that Band 1 is the linear native tRNA and that the faster migrating species in Band 2 contains the s⁴U8 x C13 crosslink, RNA from both of these bands was inspected by partial alkaline hydrolysis using 3' [³²P]-end-labeled tRNA^{Val}. After irradiation for 10 min, the RNA was separated by electrophoresis and Bands 1 and 2 were cut out and eluted (see Materials and Methods). Aliquots of the radioactive tRNA from bands 1 and 2 were partially digested by RNase T1, RNase U2, or by alkaline hydrolysis, and then run on a 10% polyacrylamide denaturing gel (Fig. 1). A complete interruption of the alkaline hydrolysis ladder occurs in the top of the gel when RNA from Band 2 was tested (Fig. 1, lanes 4 versus 3). There is some band compression in the ladders in the region corresponding to nucleotides 18-21 and in the region between A26 and A35. However, the position of A14 and G15 are clearly visible and correspond well with the alkaline hydrolysis lanes, so this compression does not interfere with the crosslink assignment. The first step missing in the interruption region is at the position of C13, indicating a crosslink to C13. The s⁴U8 is the only photoreactive nucleotide at the wavelength used for the irradiation, so band 2 must contain the s⁴U8 x C13 crosslink; its unexpected mobility on the 8 % gel must be a consequence of the small loop size and the gel electrophoresis system.

Structure of the s⁴U8 – C13 photoadduct under different conditions and temperatures

It is possible that the tRNA structure is sufficiently changed by temperature to result in changes in the photochemical reaction and a temperature dependence of product formation. To determine whether this is occurring, the identity of the photoproduct in tRNA^{fMet}, tRNA^{Val}, and tRNA^{Phe} in HiFi buffer, 50 mM Tris, pH 7.5, 30 mM KCl, 70 mM NH₄Cl, 3.5 mM MgCl₂, 8 mM putrescine, 0.5 mM spermidine, and for tRNA^{Val} in 20 mM Tris, HCl, pH 7.5, 200 mM NH₄OAc have been determined at 0 °C and 50 °C. The s⁴U8-C13 photocrosslink in tRNA made under ambient conditions is the 5,4'(pyrimidin-2'one)cytosine (Pdo (4-5)Cyd) adduct (Bergstrom et al. 1972). This product, when reduced with NaBH₄, yields Pdo(4-5)hCyd (Favre et al. 1969; 1971), which is highly fluorescent and has a strong absorbance maximum of 387 nm that distinguishes it from other dipyrimidine products (Favre and Yaniv 1971; Wang 1976). The spectra of reduced tRNA^{fMet}, tRNA^{Val}, and tRNA^{Phe} after crosslinking in HiFi buffer and reduced tRNA^{Val} after crosslinking in 20 mM Tris, HCl, pH 7.5, 200 mM NH₄OAc show a strong absorbance at 385-390 nm in all cases (Fig. 2). In addition, the presence of the isosbestic point at 355-360 nm in the spectra taken at different times of reduction (Fig. 2 A,B and G,H) indicates that only a single photoproduct is present (Favre and Yaniv 1971) at both 0 °C and 50 °C. Therefore all of the spectra indicate the Pdo(4-5)Cyd product and we can conclude that the photochemistry does not change with temperature even in the buffer without Mg²⁺. This is an indication that under all conditions the reaction proceeds through the same transition state.

Dependence of the excited state lifetimes on conditions and temperatures

For s⁴U, the photoreactive state is the T₁ state derived from the S₁ state by intersystem crossing. Excess vibrational energy of the S₁ state is dissipated immediately after photon absorption (Turro 1991) and intersystem crossing is complete for s⁴U (Favre 1990; Favre et al. 1998), so neither the formation of the S₁ or T₁ states are expected to be temperature dependent. However, there are several possible fates for the T₁ state besides photochemistry including non-radiative energy transfer, radiative de-excitation (phosphorescence), collisional quenching, and non-radiative de-excitation, which could alter the excited state lifetime.

For s^4U , the T_1 energy is lower than in other nucleotides (Favre 1990), so energy transfer from s^4U to other nucleobases is not expected. Phosphorescence has a low efficiency, the quantum yield is 1.6×10^{-3} for bulk tRNA, and, in general, is not temperature dependent (Turro 1991). It is known that s^4U can be quenched by collision by substances including halides and oxygen (Favre 1990) and this process is expected to be temperature dependent (Turro 1991). It was possible for us to test for collisional quenching in tRNA^{fMet} and tRNA^{Val} by comparing the rates of photocrosslinking in buffers prepared either with chloride or acetate as the anion. No differences in photocrosslinking rate constants were observed for tRNA^{fMet} or tRNA^{Val} in HiFi buffer which was prepared with acetate (50 mM Tris, HOAc, pH 7.5, 30 mM KOAc, 70 mM NH₄OAc, 3.5 mM Mg(OAc)₂, 8 mM putrescine, 0.5 mM spermidine) compared to the rate constants obtained for tRNA in the normal HiFi buffer prepared with chloride. On the other hand, for both of the tRNAs, in buffers containing only 200 mM NH₄⁺ or less, and no Mg²⁺ and at temperatures above 35 °C, there is about two-fold lower in apparent photocrosslinking rate in buffers containing chloride vs acetate, (data not shown). This shows that collision quenching can be a factor under conditions in which the stability of the tRNA is not complete. Since the experimental conditions for most of the experiments reported here avoid tRNA instability, collisional quenching does affect our measurement of the photocrosslinking rate constant.

Shalitin and Feitelson (1976) reported a significant and abrupt decrease in tRNA^{Val} emission (phosphorescence) as the temperature was increased. The decrease in the emission intensity could be due to an increase in the rate of non-radiative de-excitation, since this rate is about 500 times larger than that of phosphorescence and is about 100 times larger than the rate of photoreaction (Favre et al. 1979; Favre 1990). Therefore, changes in the emission intensity should be almost entirely equal to changes in the excited state lifetime and there will be more emission intensity when the s^4U has a longer excited state lifetime.

The measurements of Shalitin and Feitelson (1976) were done under different buffer conditions and did not extend over the same temperature range that we used, so new measurements have been made. Steady state emission at 510 nm, after excitation at 340 nm, was determined for tRNA^{fMet}, tRNA^{Val} and tRNA^{Phe} in HiFi buffer, for tRNA^{Val} in several additional buffers containing different

combinations of Mg^{2+} and NH_4^+ and for s^4UDP (Fig. 4). The emission of $\text{tRNA}^{\text{fMet}}$, tRNA^{Val} and tRNA^{Phe} in HiFi buffer at 298 °K are generally consistent with the published values of their T_1 lifetimes and phosphorescence quantum yields (Table 1). In order to compare the photocrosslinking rate constants for $\text{tRNA}^{\text{fMet}}$ and tRNA^{Phe} compared to tRNA^{Val} , the measured rate constants have to be corrected to reflect differences in their respective published T_1 lifetimes. The emission intensities of the tRNAs also depend on the buffer conditions which indicate changes in T_1 lifetimes. Values for the emission of tRNA^{Val} at 298 °K in the buffers used in the photocrosslinking experiments are listed in Table 1; excited state lifetimes and correction factors have been calculated from these values.

All of the tRNAs exhibit exponential decreases in emission intensity as the temperature is increased from 273 to 323 °K, indicating exponential decreases in the excited state lifetimes (Fig. 3A). Factors to correct for the effect of the differences in lifetimes on the measured rate constants for photocrosslink formation were calculated from (emission (298)/ emission (T)) using emission at 298 °K as the reference. The correction factors for temperature calculated from the emission data in HiFi buffer and in Mg^{2+} -containing buffers (Mg^{2+} concentrations greater than 0.5 mM) are all the same. The correction factors calculated for the tRNAs in buffers with NH_4OAc but without Mg^{2+} cover a larger range of values (Table 2). The correction factors for tRNA in the buffer containing 0.5 mM Mg^{2+} have intermediate values and are listed separately (Table 2). For tRNA^{Val} in 20 mM Tris, HCl, pH 7.5, 200 mM NH_4OAc , there is an abrupt decrease in emission intensity at temperatures above 30 °C probably due to collisional quenching. This is similar to the behavior reported by Shalitin and Feitelson (1976).

The exponential decrease of the emission intensity with temperature had not been noted before. Arrhenius plots of the reciprocal of the emission intensity, which indicate the rate of non-radiative de-excitation were done to determine the activation energies for the de-excitation process under different conditions (Fig. 3B). All three tRNAs in HiFi buffer and in buffers with $\text{Mg}^{2+} \geq 1$ mM have similar activation energies, 15.3 kJ mol^{-1} . For tRNA^{Val} in buffers without Mg^{2+} , the activation energy is 10.0 kJ mol^{-1} and for tRNA^{Val} in the buffer with 0.5 mM Mg^{2+} , the activation energy is 12.7 kJ mol^{-1} .

TRAP analysis photocrosslinking in tRNA^{Val} under different conditions

The stability and conformational freedom of RNA is known to depend on the concentration of both monovalent (K^+ , NH_4^+ , Cl^-) and divalent ions (magnesium and putrescine) and polyamines (spermidine) in solution (Herman and Westhof 1998; Misra et al. 2003; Koculi et al. 2004). Therefore, alterations in the monovalent ion concentration or Mg^{2+} concentration should produce differences in the activation energies which would reflect changes in either, or both, the flexibility and structure of the tRNA. To measure this effect, the temperature dependence experiments were repeated on tRNA^{Val} using buffers differing in monovalent and divalent ion concentration and identity. The first series of buffers contained 20 mM Tris, pH 7.5, 100 mM NH_4Cl and 0 to 20 mM Mg^{2+} . Another series of buffers contained 20 mM Tris pH 7.5, and 200 to 1000 mM NH_4Cl after it was determined that the tRNA was unfolding at higher temperatures in the 100 mM NH_4Cl , 0 mM Mg^{2+} buffer. The last buffer was HiFi buffer without Mg^{2+} to contrast the effects of the polyamines and magnesium.

Corrections were made to the measured rate constants to account for differences in the excited state lifetimes at 298 °K and to account for the temperature dependence of the lifetimes as described in the previous section. Plots of the rate constants versus temperature for tRNA^{Val} irradiated in these buffers again show that the rate constants increase with increasing temperature in all buffers (Fig. 4A) and Arrhenius plots show that the activation energies depend strongly on the conditions (Fig. 4B).

tRNA^{Val} thermal stability under different buffer conditions

The temperature stability of tRNA^{Val} was determined under different conditions to find out whether the tRNA was undergoing partial melting at the temperatures used in the TRAP experiments (up to 50 °C). To do this tRNA was first renatured in HiFi buffer and then was dialyzed in the cold into the desired buffer. Measurement of the A_{260} at different temperatures was used to determine the thermal stability. The measurements show that in the range between 40 and 50 °C, there is a large difference in the stability of tRNA^{Val} under the different buffer conditions even though there is only a small shift in the T_m s of tRNA^{Val} (Fig 5). Specifically, at 50 °C the fraction of the total change in A_{260} for tRNA^{Val} in the HiFi, HiFi M0, $T_{20}A_{200}$, $T_{20}A_{100}$ buffers is <3%, 4%, 4% and 21% respectively when the T_m s are 67,

71, 71 and 73 °C. This shows that there is increased flexibility in the tRNA at temperatures well below the T_m in the absence of Mg^{2+} , particularly in the buffer containing only 100 mM NH_4OAc .

METHODS AND MATERIALS

Alkaline Hydrolysis

Bands 1 and 2 containing full length native and crosslinked tRNA were cut from a preparative 8% polyacrylamide gel and eluted from gel pieces by overnight shaking in 500 mM NH_4OAc pH 4.7, 10 mM $MgCl_2$ and 2% SDS. After phenol extraction and two ethanol precipitations, portions of radioactively labeled material from the bands were treated with RNase T1, RNase U2 or alkaline hydrolysis as previously described (Juzumiene et al. 2001). Samples were resolved on a denaturing 10% polyacrylamide gel and analyzed by ImageQuant software.

Analysis of the structures of the photoproducts made at 0 and 50 °C

The structures of the photoproducts in the tRNAs were determined by the analysis described by Favre and Yaniv (1971) in which reduction of irradiated RNA yields a product with a characteristic $\lambda_{max} = 387$ nm if the Pdo(4-5)Cyd bipyrimidine photoadduct is present. To do this assay, tRNA^{fMet}, tRNA^{Val} and tRNA^{Phe} were irradiated in HiFi buffer, and tRNA^{Val} was irradiated in 20 mM Tris, pH 7.5, 200 mM NH_4OAc for 20 min at 0 °C or 50 °C. After ethanol precipitation, the RNA was redissolved in 50 mM Na cacodylate, pH 7, 100 mM NaCl and brought to pH 9.5 with NH_4OH . Spectra (450 to 300 nm) were taken of this sample and at various times after addition of $NaBH_4$ to a final concentration of 10 mM.

Determination of the phosphorescence intensity of the tRNA at different temperatures

tRNA samples were re-dissolved in HiFi buffer at concentrations of 6.5 μM and were renatured before use. For measurements in other buffers, tRNA was renatured first in HiFi buffer and then dialyzed overnight in one liter of the desired buffer in the cold, with one buffer change. Emission intensity at 510 nm was measured after excitation at 340 nm in a PTI spectrofluorimeter using a thermostatted

cuvette holder. The emission of the buffers at the same temperatures were determined separately and subtracted. A shutter was used to decrease photobleaching between measurements. The data were averages of measurements taken as the temperature was changed from 0 °C to 50 °C and then back from 50 °C to 0 °C. The emission of s⁴UDP (obtained from Sigma) was determined at a concentration of 26 μM and divided to obtain the emission for a 6.5 μM concentration.

tRNA^{Val} thermal stability under different buffer conditions

Thermal stability analysis of tRNA^{Val} under different buffer conditions was carried out in a CARY model 3 spectrophotometer. The tRNA concentrations were about 0.36 μM and measurements were made in a 1 cm cuvette at temperatures between 4 °C and 95 °C at a ramp rate of 2 °C min⁻¹ and at a rate of one reading per 0.5 °C. Denaturation and annealing measurements were done twice and data at each temperature were averaged.

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Supplemental Table 1 T_1 excited state lifetimes, phosphorescence emission intensities and correction factors

tRNA	τ_1 (μs)*	Emission 510 nm**	τ_1 used	Correction factor***
tRNA ^{fMet}	3.5	3.0	3.5	1.6
tRNA ^{Phe}	5.5-7, 6.7-7.5	10.5	7.0	0.83
tRNA ^{Val}	5.5 – 6	4.13	5.8	1.0
tRNA ^{Val} in T ₂₀ A ₁₀₀ M ₂₀		3.85	5.4	1.1
tRNA ^{Val} in T ₂₀ A ₁₀₀ M ₁₀		3.82	5.4	1.1
tRNA ^{Val} in T ₂₀ A ₁₀₀ M ₇		3.76	5.3	1.1
tRNA ^{Val} in T ₂₀ A ₁₀₀ M ₃		3.57	5.0	1.2
tRNA ^{Val} in T ₂₀ A ₁₀₀ M ₁		2.87	4.0	1.4
tRNA ^{Val} in T ₂₀ A ₁₀₀ M _{0.5}		2.43	3.4	1.7
tRNA ^{Val} in T ₂₀ A ₁₀₀₀ M ₀		3.03	4.3	1.3
tRNA ^{Val} in T ₂₀ A ₅₀₀ M ₀		2.01	2.8	2.1
tRNA ^{Val} in T ₂₀ A ₁₀₀ M ₀		1.64	2.3	2.5

* T_1 excited state lifetime (τ_1) values (Favre, 1990) were obtained in 50 mM NaCacodylate, pH 7, 50 mM NaCl, 3 mM MgCl₂; the τ_1 value for tRNA^{Phe} was also reported as 6.7 – 7.5 μ s (Favre et al. 1979).

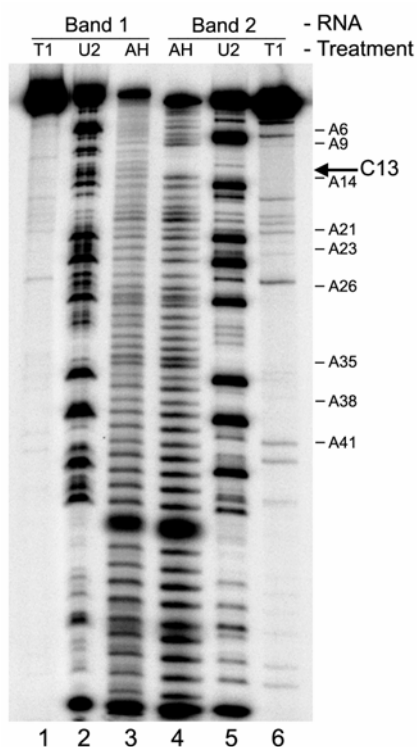
** Emission values (arbitrary units) at 298° K were obtained at 510 nm after excitation at 340 nm. tRNA^{fMet}, tRNA^{Phe} and tRNA^{Val} were measured in HiFi buffer; additional measurements of tRNA^{Val} were done in the indicated buffers. Buffers are: HiFi – 50 mM Tris, pH 7.5, 30 mM KCl, 70 mM NH₄Cl, 3.5 mM MgCl₂, 8 mM putrescine, 0.5 mM spermidine; buffers abbreviated T_xA_yM_z - x mM Tris, pH 7.5, y mM NH₄Cl and z mM MgCl₂.

*** Multiplication factor to correct measured photocrosslinking rate constants for T_1 lifetime differences relative to that of tRNA^{Val}.

Supplemental Table 2 Multiplication factors to correct measured rate constants for differences in excited state lifetimes at different temperatures relative to the lifetime at 298 °K

<u>Buffer Condition</u>	<u>Temperature (°K)</u>										
	<u>273</u>	<u>278</u>	<u>283</u>	<u>288</u>	<u>293</u>	<u>298</u>	<u>303</u>	<u>308</u>	<u>313</u>	<u>318</u>	<u>323</u>
HiFi & [Mg ²⁺] > 0.5 mM	0.592	0.658	0.730	0.811	0.900	1.00	1.110	1.233	1.370	1.521	1.689
[Mg ²⁺] = 0.5 mM	0.649	0.708	0.771	0.841	0.917	1.00	1.090	1.188	1.296	1.413	1.541
[Mg ²⁺] = 0	0.647	0.710	0.777	0.847	0.922	1.00	1.081	1.167	1.255	1.348	1.444

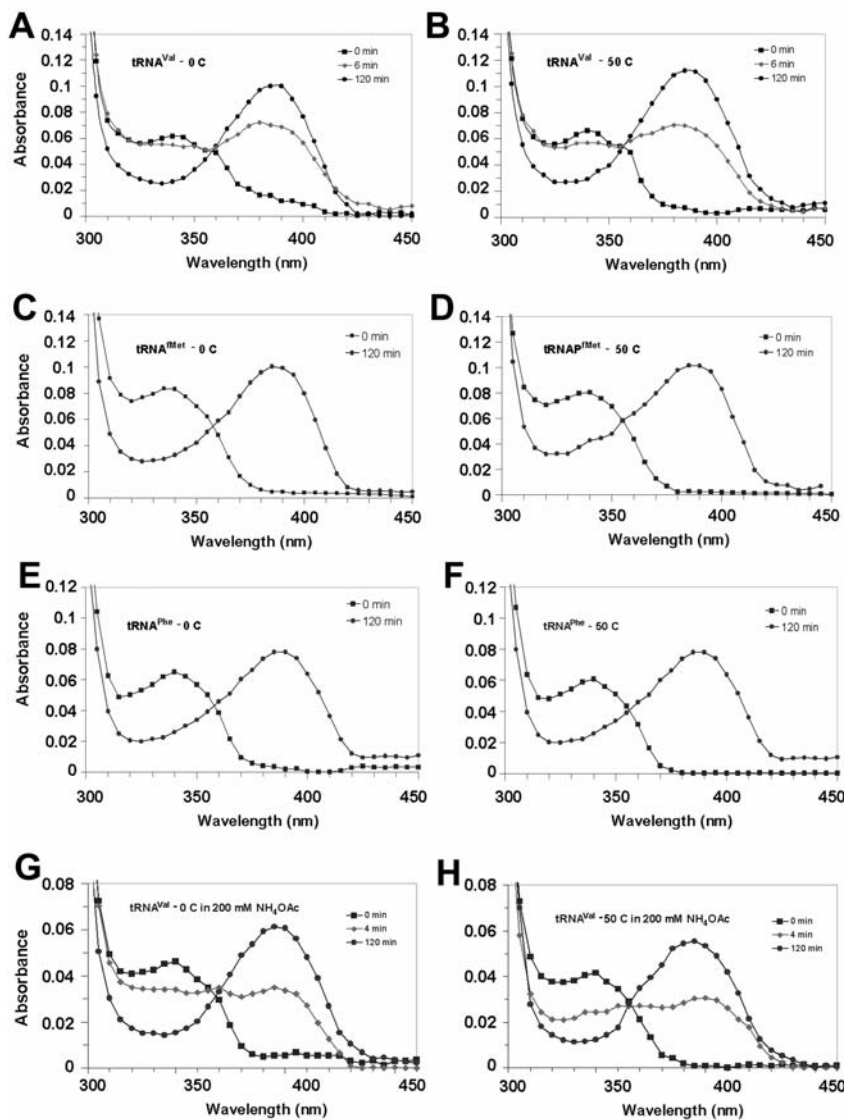
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Determination of the s⁴U8 x C13

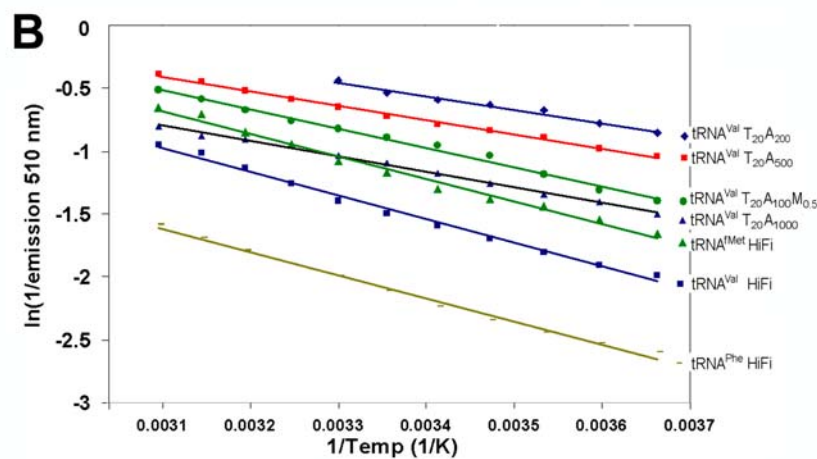
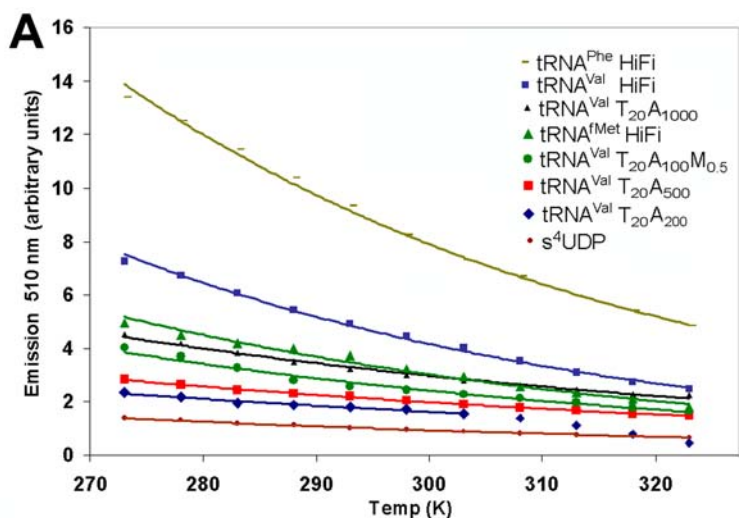
photocrosslink in the band 2 RNA. Native *E. coli* tRNA^{Val} was 3' [³²P] exchange-labeled and irradiated as described for 10 min at 0 °C. The RNA was separated on an 8% denaturing polyacrylamide gel and portions of the gel containing the linear uncrosslinked (band 1) and crosslinked (band 2) tRNA were cut out. RNA was eluted by shaking overnight. Portions of the recovered radioactive RNA from both bands were treated with RNase T1, RNase U2, or subjected to partial alkaline hydrolysis and the products separated on a 10% denaturing polyacrylamide gel. RNase T1 and U2 cut on the 3' side of G and A residues respectively, and the sequence is determined

according to that pattern in the alkaline hydrolysis lanes. The interruption of the ladder in lane 4 (alkaline hydrolysis of RNA from band 2) occurs one position above A14, indicating a crosslink to C13.



Supplemental Figure 2. Spectral analysis of the reduction of photocrosslinked tRNA to determine if the same photoproduct is made at all temperatures. tRNAs were irradiated in HiFi buffer (panels A-F) or 200 mM NH_4OAc (panel G and H) at 0 °C or 50 °C for twenty minutes. They were precipitated and re-dissolved in 50 mM NaCacodylate, pH 7.0, 100 mM NaCl. NH_4OH was added to bring the pH to pH 9.5 and then NaBH_4 was added to reach a final concentration of 10 mM. All reductions were done at 22 °C.

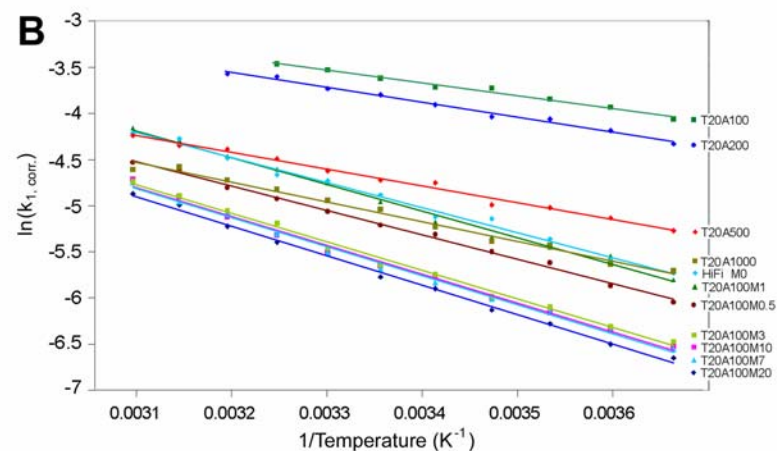
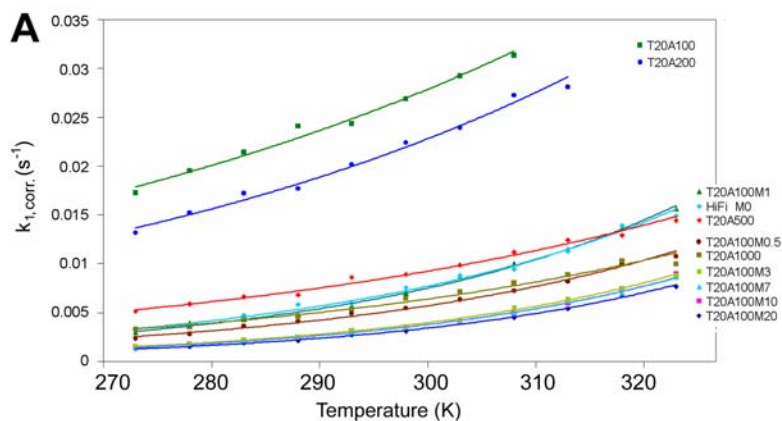
Spectra were recorded at the indicated times. Panels A, C, E and G contain spectra recorded from tRNA crosslinked at 0 °C and panels B, D, F and H contain spectra recorded from tRNA crosslinked at 50 °C. The strong fluorescence maxima at 386 nm and the occurrence of the isosbestic point at 358 nm during the time course of reduction are characteristic of the reduction exclusively of the 5,4'-(pyrimidin-2'-one)cytosine (PdO(4,5)Cyd) photoproduct. (Favre 1991; Wang 1976).



Supplemental Figure 3.

Temperature dependence of steady state emission intensity of tRNA and s⁴UDP. A. Emission at 510 nm for tRNA^{Phe}, tRNA^{fMet} and tRNA^{Val} after excitation at 340 nm. tRNA samples were renatured in HiFi buffer and dialyzed into the indicated buffers in the cold before analysis. tRNA concentrations were approximately 6.5 μM; the s⁴UDP concentration was 26 μM and the emission intensity was divided for comparison. The emission of the buffer at each

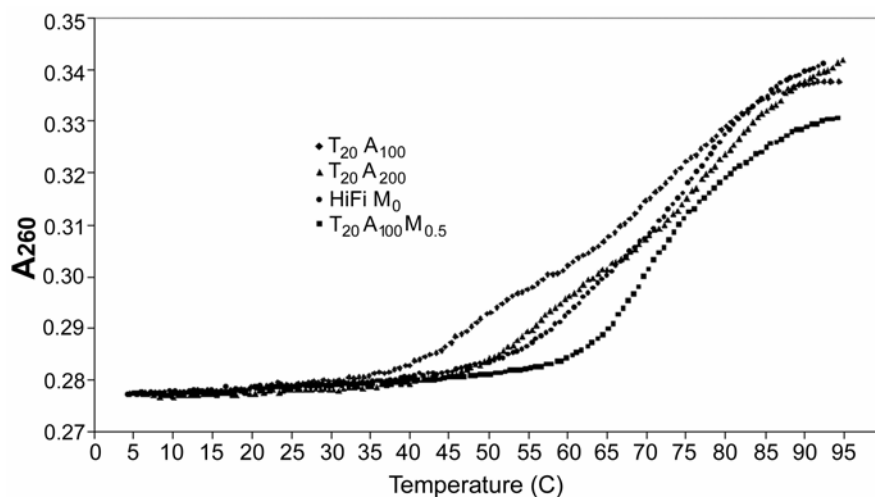
temperature was subtracted from the measurements and values were corrected to the same tRNA concentration, calculated from the A₂₆₀ of the sample. The fitted lines through the data are exponential curves and are fitted to all data except for tRNA^{Val} in 20 mM Tris, 200 mM NH₄OAc, which has non-exponential behavior at temperatures above 30 °C. B. Arrhenius analysis of the de-excitation process. The natural logarithm of the reciprocal emission intensity, which is proportional to the rate of non-radiative de-excitation was plotted versus inverse temperature to determine the activation energy for the de-excitation process. Linear behavior is seen in all cases except for tRNA in 10 mM Tris, pH 7.5, 200 mM NH₄OAc above 35 °C.



Supplemental Figure 4.

Temperature dependence of rate constants for formation of the s⁴U8 x C13 crosslink in tRNA^{Val} under different conditions. A. First order rate constants calculated from one min irradiations at different temperatures and corrected for changes in excited state lifetimes. Average values are plotted versus temperature. The fitted lines are exponential dependence on temperature. B. Arrhenius plots of corrected first order rate constants.

The fitted lines are linear dependence of the natural logarithm of the rate constants on the inverse temperature. All data were used for fitting except for tRNA^{Val} in 20 mM Tris, pH 7.5, 100 mM NH₄Cl and in 20 mM Tris, pH 7.5, 200 mM NH₄Cl, where first order rate constants deviated significantly from expected values at 40 – 50 °C and at 45 – 50 °C.



Supplemental Figure 5. Melting profiles for tRNA^{Val} under different conditions. Absorbance measurements were done in buffers containing 20 mM Tris, pH 7.5, 100 mM NH₄Cl, 20 mM Tris, pH 7.5, 200 mM NH₄Cl, HiFi buffer with 0 mM Mg²⁺ or containing 20 mM Tris, pH 7.5, 100 mM NH₄Cl, 0.5 mM Mg²⁺ as indicated in the figure. The values are from three experiments and were normalized to a starting absorbance of 0.277. The T_ms of the tRNA under these conditions are about 67 °C in T₂₀A₁₀₀, 71 °C in T₂₀A₂₀₀, 71 °C in HiFi M₀, and 73 °C in T₂₀A₁₀₀M_{0.5}.