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An ultraviolet light-induced crosslink in yeast tRNA^{Phe}

Linda S.Behlen, Jeffrey R.Sampson+ and Olke C.Uhlenbeck*
Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, CO 80309-0215, USA

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ABSTRACT

The irradiation of native or unmodified yeast tRNA^{Phe} by short wavelength UV light (260 nM) results in an intramolecular crosslink that has been mapped to occur between C48 in the variable loop and U59 in the T loop. Photo-reversibility of the crosslink and the absence of fluorescent photo adducts suggest that the crosslink product is a cytidine-uridine cyclobutane dimer. This is consistent with the relative geometries of C48 and U59 in the crystal structure of yeast tRNA^{Phe}. Evaluation of the crosslinking efficiency of the mutants of tRNA^{Phe} indicates that the reaction depends on the correct tertiary structure of the RNA.

INTRODUCTION

The formation of UV induced crosslinks, either within (1–7) or between (8,9) RNA molecules has been useful in defining constraints in the folding of RNA chains. While crosslinks have been reported between a variety of pairs of nucleotides, the exact chemical nature of the crosslink has only been determined for the Cyt(5–4)Pyo adduct formed between s^{4}U8 and C13 of *E. coli* tRNA^{Val} (2) and a cyclobutane dimer that forms between cmo^{5}U34 of *E. coli* tRNA^{Val}1 and C1400 of *E. coli* 16s rRNA (10).

In this paper we describe an efficient photocrosslink in unmodified yeast tRNA^{Phe} upon irradiation with short wavelength ultraviolet light. The crosslink was found to occur between two pyrimidines 11 nucleotides apart in the primary sequence, but held in close proximity by the tRNA tertiary structure. This suggested that the efficiency of the crosslinking reaction might reflect the degree to which the tRNA^{Phe} folded correctly. Rates of UV crosslinking on a variety of well-characterized tRNA^{Phe} mutants were determined to test this assumption.

MATERIALS AND METHODS

Wild type and mutant unmodified yeast tRNA^{Phe}s were prepared by *in vitro* transcription with T7 RNA polymerase (11). Internally labeled molecules were prepared with [α-^{32}P]-CTP (150 Ci/mMole). 5′-^{32}P-labeled RNAs were obtained by dephosphorylating the transcript with calf intestinal phosphotase and phosphorylating with [γ-^{32}P]-ATP and polynucleotide kinase. 3′ end-labeled transcripts were prepared by addition of [5′-^{32}P]-pCp (300 Ci/mM) to the tRNA with T4 RNA ligase (12).

Crosslinking reactions were carried out in untreated microtiter plates in 40 μl reactions containing 1 μM tRNA, 10 mM Tris-HCl (pH7.6), 15 mM $MgCl_2$, and 25 mM KCl. All tRNAs were heated to 80°C in the absence of $MgCl_2$ and slow cooled prior to addition of $MgCl_2$ to assure a native conformation. A mercury germicidal lamp (model UVG-11, 14,000 μwatts/cm^2 at a distance of 7.62cm, UVP Inc., San Gabriel, CA) with a peak intensity at 254 nM was used as the light source. The lamp was suspended 2 cm from the top of the plate and the temperature was maintained at 25°C. Five microliter aliquots were removed at the indicated times and the products of the reaction were analysed on 15% polyacrylamide, 7M urea gels. The fraction of crosslinking was determined by digital quantitation of gels on a Molecular Dynamics Phosphor Imager. Crosslinking rates with a standard deviation of approximately 0.006 min^{-1} were obtained from semi-log plots over the first 15% crosslinking.

To map the crosslink, 3′ or 5′ end-labeled RNA was irradiated for 20 min. The crosslinked RNA was then purified on a gel, dissolved in 10 mM $(NH_4)_2CO_3$ buffer (pH 9) and heated to 95°C for 5 min. The reaction was then applied to a 15% denaturing polyacrylamide gel with uncrosslinked tRNA that had been subjected to partial T1 or alkali digestion.

Lead cleavage rates were determined as described in Behlen et al. (13).

RESULTS AND DISCUSSION

When unmodified yeast tRNA^{Phe} is irradiated with short wavelength UV light, two distinct photoproducts could be detected on denaturing polyacrylamide gels (Fig. 1A). The major product, P1, appears with an initial rate of 0.06 min^{-1} and continues to accumulate for about 30 min. (Fig. 1B). A smaller amount of a slower migrating photoproduct, P2, forms at a slower rate after a short lag. After 40 min., 30% native tRNA^{Phe} remains and no further reaction appears to occur. No photoproducts were observed when irradiation was carried out in a low ionic strength buffer containing 3.5 M urea where the tertiary structure of tRNA is disrupted (Fig. 1A). When 3′-^{32}P-

* To whom correspondence should be addressed

+ Present address: Division of Biology 147-75, California Institute of Technology, Pasadena, CA 91125, USA

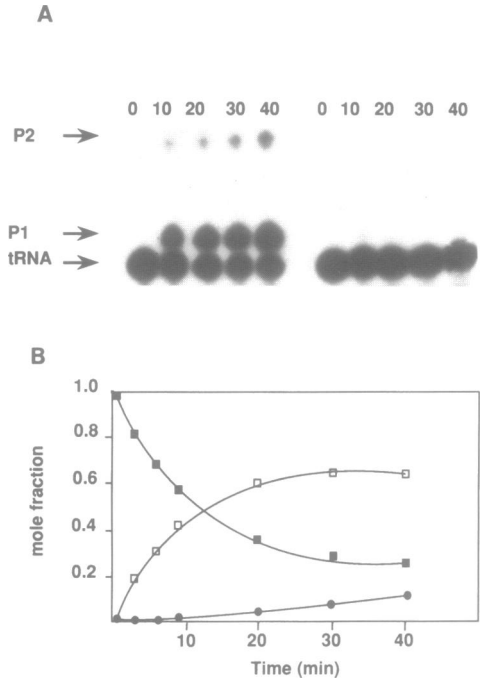

Figure 1. UV crosslinking of $tRNA^{Phe}$. **(A)** Time course of [α-^{32}P]-CTP labeled $tRNA^{Phe}$ upon exposure to shortwave UV light. Numbers above lanes refer to minutes of exposure. Left, 1μM tRNA, 10mM Tris-HCl (pH 7.6), 15mM $MgCl_2$ and 25mM KCl. Right, same except with 3.5M urea and no $MgCl_2$. **(B)** Kinetics of crosslinking reaction $tRNA^{Phe}$ (n), P1 (q), P2 (l).

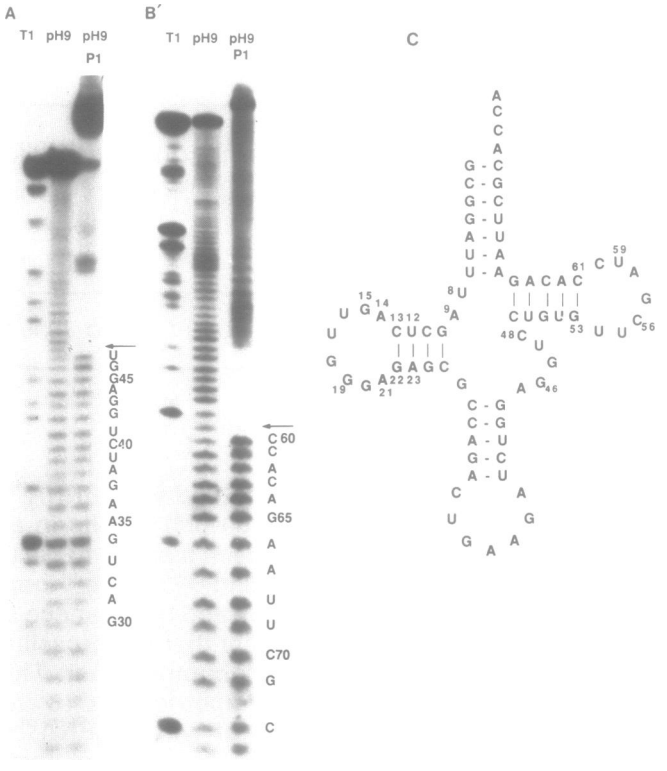

Figure 2. Identification of the crosslinked residues. **(A)** Mapping of the 5′ site. 5′-^{32}P- labeled $tRNA^{Phe}$ was partially digested with RNase T1 (T1) or partially hydrolyzed with alkali (pH 9). 5′-^{32}P-labeled P1 was partially hydrolyzed with alkali (pH 9 P1). **(B)** Mapping of the 3′ crosslink site as in panel A with 3′-^{32}P-labeled P1 and tRNA. **(C)** $tRNA^{Phe}$ cloverleaf showing the location of the crosslink and sites of mutagensis.

labeled modified yeast $tRNA^{Phe}$ was used instead of the transcript, the same pattern of photoproducts accumulated at similar reaction rates. Thus, the fourteen nucleotide modifications present in yeast $tRNA^{Phe}$ do not appear to affect the reaction.

Since total ribonuclease T1 digestion of the major photoproduct indicated the formation of a unique crosslink between two oligonucleotides (data not shown), a crosslink mapping experiment was performed (Fig. 2). Partial alkaline hydrolysis of 5′-^{32}P-labeled P1 revealed a gap in the ladder at a unique site in the molecule (panel A). The gap is the result of the much slower mobility of alkaline hydrolysis products that contain the crosslink. Comparison with partial alkaline and RNase T1 digestion products of 5′-^{32}P-labeled uncrosslinked tRNA identifies the last nucleotide before the gap as U47, making C48 the 5′ nucleotide of the crosslink. The corresponding experiment using 3′-^{32}P-labeled RNA (panel B) identified the 3′ nucleotide of the crosslink as U59. Similar mapping experiments performed with the crosslinked product obtained with modified $tRNA^{Phe}$ revealed the identical crosslink. Since no evidence could be found for additional crosslinks in P1 by either partial alkaline hydrolysis or total T1 digestion, we conclude that the primary photoproduct P1 is a tRNA containing a C48-U59 crosslink. It is noteworthy that alkaline hydrolysis of the end-labeled linear RNAs remaining after irradiation did not reveal any crosslinking gaps (data not shown). This indicates that the linear tRNA fraction does not contain other photocrosslinks which did not separate on the gel.

The C48-U59 crosslink can be reversed upon additional irradiation with short wavelength UV light. When P1 is purified, renatured, and subjected to further UV treatment, it is converted to roughly equal amounts of P2 and linear tRNA (Fig. 3A).

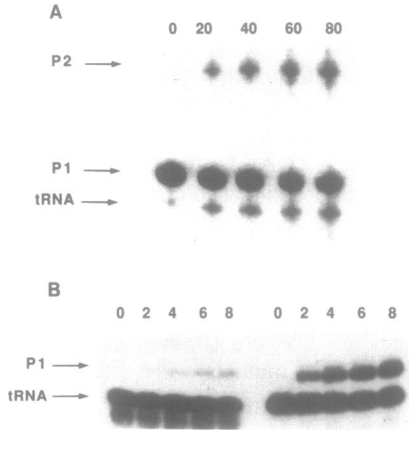

Figure 3. Photoreversal and subsequent recrosslinking of P1. **(A)** Gel purified [α-^{32}P] CTP labeled P1 exposed to shortwave UV light. Numbers above lanes refer to the time of exposure in min. **(B)** Left, recrosslinking of [α-^{32}P]-CTP labeled P1 linearized by exposure to shortwave UV light for 15 min. under the same denaturing conditions as in figure 1A. Right, crosslinking of $tRNA^{Phe}$. Minutes of exposure are indicated above the lanes.

Quantitation of the gel gives a rate of formation of P2 of 3.0×10^{-3} min^{-1} and a rate of conversion back to linear tRNA of 2.2×10^{-3} min^{-1}. Since the rate of formation of P2 from P1 is much faster than the rate of P2 formation starting with linear

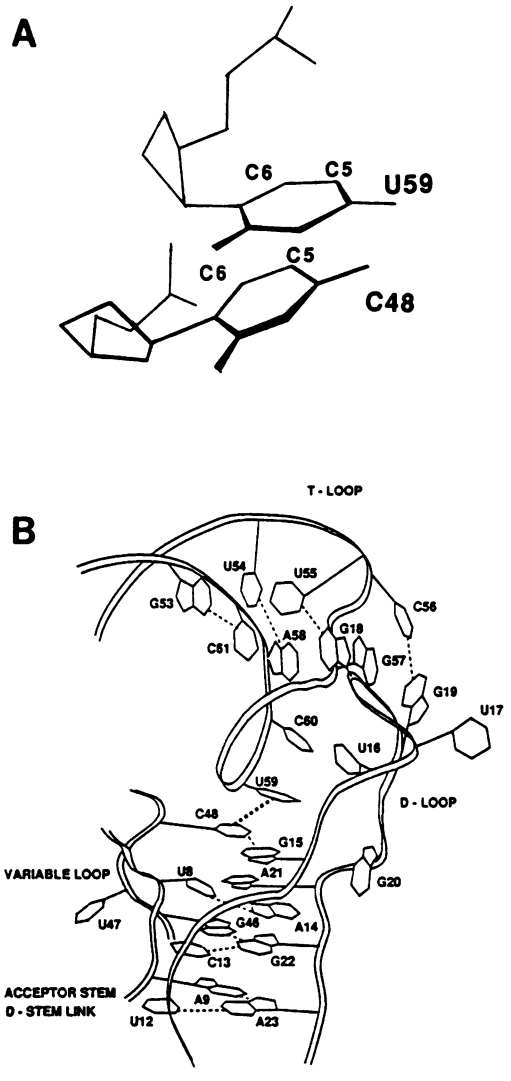

Figure 4. UV crosslinked nucleotides and surrounding $tRNA^{Phe}$ structure. **(A)** Orientation of the crosslinked nucleotides in the crystal structure. **(B)** The folded structure of $tRNA^{Phe}$, adapted from the crystal structure, in the vicinity of the crosslink. A ribbon represents the phosphodiester backbone. Functional groups have been omitted from pyrimidine and purine rings for clarity. The crosslink between U59 and C48 is represented by a single heavy dashed line, while pairing between residues is indicated by the lighter dashed lines; however, these are not intended to represent actual bonds.

tRNA (Fig. 1), P1 appears to be an intermediate in the formation of P2. The rate of conversion of P1 back to linear tRNA is approximately 3−1/2 times slower than its rate of formation from linear RNA under identical irradiation conditions. The equilibrium between forward and reverse reactions may account for the reaction in Fig. 1 reaching about 70% crosslinking.

In an effort to determine whether the formation of $tRNA^{Phe}$ from P1 by irradiation represented true photoreversal, a sample of P1, prepared by irradiating $tRNA^{Phe}$ for 30 min., was purified, renatured, and re-irradiated for 90 min. The $tRNA^{Phe}$ fraction from this second irradiation was gel purified, renatured and irradiated a third time to see whether it would crosslink again. Very little recrosslinking was observed even after 30 min. of irradiation (data not shown). This suggested that the formation of $tRNA^{Phe}$ from P1 either did not represent true photoreversal or that the extensive irradiation used to prepare the $tRNA^{Phe}$ resulted in additional photodamage that prevented recrosslinking. In order to distinguish between these possibilities, a protocol involving much less total irradiation time was used. P1 was prepared by irradiating for only 15 min. After purification, photoreversal was carried out under denaturing conditions. This prevents any recrosslinking or formation of P2 and therefore results in sufficient amounts of $tRNA^{Phe}$ after only 15 min. of irradiation. When this preparation of $tRNA^{Phe}$ was gel purified, renatured and subjected to irradiation, clear recrosslinking occurred although the rate is about eight times slower than $tRNA^{Phe}$ that had not been irradiated (Fig. 3B). This experiment clearly shows that the $tRNA^{Phe}$ prepared by photo reversal is competent to be crosslinked a second time and therefore suggests that the structures of C48 and U59 are restored after photoreversal. However, the slower rate of recrosslinking suggests that a considerable fraction of the molecules have undergone photodamage even with this milder irradiation procedure. This photodamage does not necessarily have to occur at C48 or U59 since, as will be shown below, structural alterations elsewhere in $tRNA^{Phe}$ can reduce photo crosslinking rates.

There are two different classes of pyrimidine-pyrimidine dimers that can form upon irradiation of nucleic acids with ultraviolet light (14, 15). There are several reasons why we believe that the C48-U59 crosslink belongs to the cyclobutane dimer class rather than the class which involves a single bond between the 4 carbon of one pyrimidine and the 5 carbon or 6 carbon of the other. First of all, many of the single bond adducts are fluorescent, either directly or after reduction with $NaBH_4$ (2, 15, 16). We were not able to detect any fluorescence in P1, either before or after $NaBH_4$ treatment. Secondly, although the single bond dimers form through a photolabile oxetane intermediate, the intermediate is acid labile and rarely accumulates in large amounts (17, 18). Since treatment of P1 with 0.1 M HCl at 25ØÓC for 15 min. does not cause breakdown or affect photoreversal, oxetane formation does not account for the P1 crosslink. Finally, the ability of the C48-U59 crosslink to undergo true photoreversal is a property of cyclobutane pyrimidine dimers. The well characterized cyclobutane dimers in double stranded DNA (19) and the crosslink between cmo^5 U34 of $tRNA^{Val}$ and C1400 of *E. coli* 16S rRNA (10) are both found to be photoreversible.

The crystal structure of yeast $tRNA^{Phe}$ is consistent with the formation of the C48-U59 cyclobutane dimer. The two pyrimidine rings are adjacent to one another in the folded structure and their 5−6 double bonds are nearly parallel and 4.6 to 4.7 Å apart (Fig. 4A). Of the four possible stereo configurations of the cyclobutane dimer, the tRNA structure predicts that the two pyrimidine rings are on the same side of the plane formed by the cyclobutane ring in the *cis* configuration and *syn* in that the cyclobutane ring is formed by bonding carbon 5 of U48 to carbon 5 of C59 and carbon 6 of U48 to carbon 6 of C59. We have, however, not confirmed that the product has this *cis-syn* configuration. It is important to note, however, that it would have been difficult to predict from the tertiary structure that the C48-U59 dimer would form significantly more efficiently than other possible photodimers. Several of the adjacent pyrimidines in the helical regions of $tRNA^{Phe}$ have their 5−6 double bonds in somewhat closer proximity, although generally not quite as parallel. For example, the 5−6 double bonds of U8 and C13, residues which form a crosslink upon exposure to long wave UV light in tRNAs containing S^4U8 (2), are also roughly parallel and 4.7 Å apart yet no U8-C13 cross link is detected. Thus,

Table 1.

Mutation	Relative Crosslinking Rate[a]	Relative Lead Cleavage Rate[b]
U59C	0.39	0.10
G15C	1.0	0.14
G15A	1.0	0.85
C48U	0.09	0.42
G15A,C48U	0.13	1.2
C48G	<0.01	0.21
C56G	0.17	0.07
C56G,G19C	1.0	1.0
G46C	0.33	0.22
G46A,G22A,G13U	0.99	0.44
G53C,C61G	0.44	0.08
A9U	0.95	0.18
U8C	0.89	0.10
A21C	0.82	0.14

[a] Rate of crosslinking was normalized to 0.06 min^{-1} for the wild type transcript.
[b] Data taken from reference 13 or determined here.

cyclobutane dimer formation is not related to the distance or orientation of the pyrimidines in a simple way. The identity of the participating nucleotides, their environment and the local flexibility of the chain could potentially affect both the forward and reverse reaction rates, making it difficult to predict both the rate and extent of crosslink formation.

The juxtaposition of C48, in the variable loop, and U59, in the T loop, are maintained by the large number of tertiary interactions involved in the folding of the tRNA (Fig. 4B). One might expect that mutations of $tRNA^{Phe}$ which disrupt tertiary structure would reduce crosslinking efficiency. Thus it may be possible to use the rate of formation of the C48-U59 crosslink as a means to evaluate the structural integrity of a given $tRNA^{Phe}$ mutant. We have previously shown that another specific reaction involving $tRNA^{Phe}$, chain cleavage at U17 by lead, is such a useful 'folding assay' (13). The rate of lead cleavage was used to distinguish between mutants of yeast $tRNA^{Phe}$ that were inactive in aminoacylation because of incorrect folding from those which were inactive due to the loss of contact between enzyme and tRNA (20, 21). To examine the sensitivity of photocrosslinking to $tRNA^{Phe}$ tertiary structure and to possibly establish a second 'folding assay', we measured the initial rate of photocrosslinking for a variety of available $tRNA^{Phe}$ mutants. The location of the nucleotides that were mutated in the central core of yeast $tRNA^{Phe}$ are shown in the diagram in Fig. 4B. For sake of comparison, the crosslinking rates are normalized in Table 1 to that of wild type transcript. In addition, the relative rates of lead cleavage for each mutant (13) are included. Since U59 also forms part of the lead binding pocket (22), both reactions occur in the same part of the $tRNA^{Phe}$ structure and thus might be expected to show a similar sensitivity to mutation.

We will first consider mutations of the two nucleotides which crosslink. U59 is stacked within the $tRNA^{Phe}$ tertiary structure, but does not form any hydrogen bonds with other parts of the molecule. While the U59C mutation reduces the rate of lead cleavage by preventing proper lead binding (13), the change is not believed to alter the folding of $tRNA^{Phe}$ since aminoacylation proceeds at a normal rate (20). The observation that U59C shows a 2.5-fold lower rate of crosslinking suggests that the intrinsic photo-reactivity of C is less than U at this position within the $tRNA^{Phe}$ structure. Considerable difference in the rate of photodimer formation among pyrimidine pairs has also been observed in double stranded DNA (23).

Analysis of mutations of the other crosslinked nucleotide, C48, is potentially complicated by the fact that it forms a *trans* base pair with G15 in the $tRNA^{Phe}$ structure that might be important for constraining the cytidine ring in the correct conformation for crosslinking. Crosslinking to C48 is unaffected by both the G15A and G15C mutations. This is not surprising in the case of G15A since this mutant also cleaves with lead at a near normal rate. However, G15C cleaves with lead at a reduced rate, implying an altered structure. Perhaps the native tRNA conformation at residue 48 is not optimal for crosslinking so that the increased flexibility resulting from the G15 mutation thereby allows rapid crosslinking. As would be expected, when C48 is changed to an unreactive G, no crosslinking is observed. It is interesting that when C48 is changed to the more reactive uracil (24), crosslinking is decreased 11 fold. It is possible that this decrease in the efficiency of crosslinking is due to the disruption of the tertiary interaction with G15 since the lead cleavage rate is also reduced for this mutant. The double mutant, G15A C48U, is expected to form the A15-U48 pair seen in 13% of tRNAs (25) and is found to cleave normally with lead. When this altered tertiary interaction is irradiated, the rate of crosslinking is still 8 fold slower than wild type. Either the alternate tertiary pairing results in a subtle structural change that effects crosslinking and not lead cleavage or the identity of the nucleotide participating in the crosslink effects the reaction rate in some other way.

A number of mutations of nucleotides which participate in the tertiary folding of tRNA were tested for crosslinking (Table 1). Disruption of the tertiary G19-C56 base pair with the C56G mutation reduces crosslinking substantially despite the fact that the base pair is 15Å away from the site of crosslinking. This is clearly a result of misfolding since inclusion of the compensating G19C mutation restores crosslinking to the normal rate. Similarly, the G46C mutation reduces crosslinking three-fold as a result of disrupting the C13-G22-G46 tertiary interaction. The alternate U13-A22-A46 tertiary interaction crosslinks normally. Finally, changing the G53-C61 base pair to C53-G61 reduces the crosslinking rate by one half. This mutation is believed to disrupt the folding the $tRNA^{Phe}$ by preventing the formation of the hydrogen bond between the amino group of C61 and the non-bridging oxygen of phosphate 60 (13, 26). Since all these results correlate well with the lead cleavage rate, we can conclude that disrupting the tertiary structure of tRNA can clearly reduce crosslinking efficiency.

Analysis of crosslinking in three other mutants of $tRNA^{Phe}$ suggests, however, that crosslinking is not equally sensitive to all disruptions of tertiary structure. The A9U mutation, which disrupts the A9-U12-A23 triple 13.5 Å away from the crosslink, shows a normal crosslinking rate, despite the fact that a five-fold reduction in the lead cleavage rate is observed for the same mutation. Similarly, the U8C and A21C mutations show little change in crosslinking rates despite the fact that they are fairly close to the crosslinking site and show reduced rates of lead cleavage. Thus, the crosslinking reaction appears in general to be somewhat less sensitive than lead cleavage in its ability to detect distant defects in the overall folding of $tRNA^{Phe}$.

While it is clear that the C48-U59 crosslink accurately reports on the close proximity of the two nucleotides in the folded tRNA structure, it is difficult to relate crosslinking efficiency to perturbations on the structure in a simple way. To begin with,

the apparent rate of crosslinking reflects the sum of several different reaction rates that differ in their sensitivity to structural changes. While the forward photochemical reaction must require proximity and proper alignment of C48 and U59, the precise requirements are unknown and likely to be subtle since several other potential pyrimidine photodimers do not form. As discussed above, it is quite possible that the flexibility of the two pyrimidines to adopt an optimal configuration for crosslinking may be an important factor. The reverse photochemical reaction is unlikely to be very sensitive to structural perturbations since it proceeds well when the tRNA is denatured. Finally, there appears to be a substantial contribution of one or more photochemical side reactions that were found to dramatically reduce crosslinking after as little as 30 min. of exposure to UV light. It is not known how much these side reactions will change with mutant tRNAs.

A second factor that complicates a simple structural explanation for changes in UV crosslinking rate of tRNA mutants is that we do not know how much a given mutant alters the structure and dynamics of the tRNA molecule in the region of residues 48 and 59. It is quite likely that each mutant will disrupt the structure to a differing extent resulting in widely variable changes in crosslinking efficiencies. This problem complicates the interpretation of the lead cleavage reaction in the same way and means that both reactions are best regarded as 'assays' of $tRNA^{Phe}$ structure and dynamics that report on structural changes in a highly idiosyncratic manner.

The C48-U59 crosslinking 'assay' is therefore expected to be useful in evaluating $tRNA^{Phe}$ folding in a manner quite similar to that of the lead cleavage reaction. A crosslinking rate is easy to determine and, if similar to wild type $tRNA^{Phe}$, suggests that the molecule must fold similar to $tRNA^{Phe}$. This will not only be useful for examining mutants of $tRNA^{Phe}$, but also in the analysis of $tRNA^{Phe}$-like structures that were obtained as a result of *in vitro* selection experiments (27).

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