Structural Elements That Contribute to an Unusual Tertiary Interaction in a Transfer RNA†

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ABSTRACT: Transfer RNAs (tRNAs) contain a set of defined tertiary hydrogen-bonding interactions that are established between conserved and semiconserved nucleotides. Although the crystal structures of tRNAs describe each of the tertiary interactions in detailed molecular terms, little is known about the underlying structural parameters that stabilize the tertiary interactions. *Escherichia coli* (*E. coli*) $tRNA^{Cys}$ has an unusual tertiary interaction between G15 in the dihydrouridine (D) loop and G48 in the variable loop that is critical for cysteine aminoacylation. All other tRNAs have a purine 15 and a complementary pyrimidine 48 that establish a tertiary interaction known as the Levitt base pair [Levitt, M. (1969) *Nature 224*, 759–763; Klug et al. (1974) *J. Mol. Biol. 89*, 511–516]. In this study, the G15·G48 tertiary interaction in *E. coli* $tRNA^{Cys}$ was used to investigate the structural elements that contribute to its variation from the Levitt base pair. Analysis with chemical probes showed that substitution of U21 with A21 in the D loop and formation of a Watson–Crick base pair between nucleotides 13 and 22 in the D stem switch the hydrogen-pairing of G15·G48 to a Levitt-like G15·G48 base pair. This switch was accompanied by a decrease of the catalytic efficiency of aminoacylation by 2 orders of magnitude. In contrast, insertion of additional nucleotides in the D or variable loops had little effect. The results shed new light on the significance of A21 in tRNA structure, and suggest that the ability to establish tertiary interactions at U8·A14·A21 and at positions 13·22·46 that enhances the stacking interactions in the D-anticodon helix is a major determinant for the tertiary interaction between positions 15 and 48.

All tRNAs fold into an "L"-shaped structure through tertiary hydrogen interactions (Rich & RafBhandary, 1976). In the "L"-shaped three-dimensional structure of yeast $tRNA^{Phe}$, a G15·C48 Levitt base pair stacks with a U8·A14 reverse-Hoogsteen base pair to stabilize the joining of two long helical stems of L, the acceptor–TΨC stem and the D-anticodon stem (Kim *et al.*, 1974a; Robertus *et al.*, 1974; Ladner *et al.*, 1975; Quigley & Rich, 1976). The stacking interaction is such that G15 is protected from chemical probes. Studies with yeast $tRNA^{Phe}$, and with several others, have shown that the N7 of purine 15 is not reactive with the chemical probe dimethyl sulfate (DMS)[1] (Theobald *et al.*, 1988; Puglisi *et al.*, 1993). However, G15 in *Escherichia coli* $tRNA^{Cys}$ is reactive with DMS (Hou *et al.*, 1993). The reactivity with DMS suggests an alteration of the backbone structure at G15 such that it disrupts the stacking interaction of G15 with A14. Analysis of this tRNA with the chemical probe kethoxal suggests that G15 can form two hydrogen bonds with G48 to establish a tertiary interaction that involves protons donated from the exocyclic N2 and accepted by the guanine ring N3 (Hou *et al.*, 1993). This hydrogen donor–acceptor scheme has not been found in any other existing RNA structures, and is quite distinct from the predicted Levitt G15·G48 tertiary interaction, which would involve a hydrogen donor from N1 and a hydrogen acceptor of O6 (Figure 1). Thus, the unusual G15·G48 tertiary interaction in *E. coli* $tRNA^{Cys}$ is a variation of the Levitt base pair, and provides an opportunity in investigate the structural basis that contributes to this base pair.

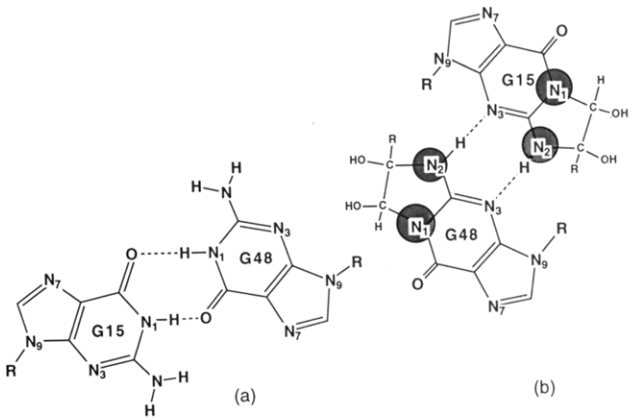

FIGURE 1: Proposed tertiary hydrogen-bonding interactions between G15 and G48 that mimic the Levitt base pair (a) or hydrogen interactions that involve a novel pairing scheme that was proposed for G15·G48 in *E. coli* $tRNA^{Cys}$ (b) (Hou *et al.*, 1993). Only the (b) configuration is capable of reacting the N1 and N1 hydrogens of guanines with kethoxal, $RC_2(H)_3(OH)_2$, to form a five-membered ring adduct (shaded in circles).

The unusual G15·G48 base pair in *E. coli* $tRNA^{Cys}$ has a critical role in the specific aminoacylation by *E. coli* cysteine–tRNA synthetase (Hou *et al.*, 1993; Hou, 1993). Alteration of this base pair to G15·C48 or C15·G48 essentially eliminates aminoacylation *in vitro*, while substitution of this base pair with G15·U48 or U15·G48 retains partial activity. The dependence of aminoacylation on G15·G48 is probably related to the unique backbone structure of G15. Structural modeling shows that the sugar–phosphate backbone at position 15 in the partially active mutants is aligned with that in the wild type, but substantially altered in the defective mutants (Hou *et al.*, 1993). It should be noted that in the Levitt configuration, alteration of nucleotides within the confines of base comple-

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[1] Abbreviations: DMS, dimethyl sulfate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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mentarity does not have a significant effect on aminoacylation (Sampson *et al.*, 1990).

To elucidate the structural basis in *E. coli* $tRNA^{Cys}$ that contributes to the unusual G15·G48 base pair, the rationale was to introduce site-directed nucleotide substitutions to the tRNA and look for substitutions that switch the unusual G15·G48 base-pairing to the Levitt configuration. The nucleotide substitutions that confer the switch of base-pairing were interpreted as the structural elements that were required for the unusual base pair. The two types of base-pairings can be distinguished by chemical probes and by *in vitro* aminoacylation with cysteine–tRNA synthetase. The present studies show that substitution of U21 with A21 in the D loop and creation of a complementary U13·A22 base pair that replaces the mismatched A13·A22 collectively switch the unusual G15·G48 to a Levitt-like base pair. The switch is manifested by changes of hydrogen bonds and by an enhanced stacking interaction between G15 and A14 such that the N7 of G15 is protected from DMS. The switch results in decreased aminoacylation of *E. coli* $tRNA^{Cys}$, and confirms the dependence of the cysteine enzyme on the unusual G15·G48 for aminoacylation. The results were interpreted in the framework of stacking interactions in the crystal structure of tRNAs, and provided some insight into the predictions of RNA structure.

MATERIALS AND METHODS

Construction of tRNA Mutants. Plasmid derivatives of pTFMa that encode the genes for mutants of *E. coli* $tRNA^{Cys}$, flanked by the T7 RNA polymerase promoter and a *Bst*NI restriction site, were prepared by procedures described previously (Hou *et al.*, 1993; Putz *et al.*, 1991; Sampson *et al.*, 1988). Transcription of tRNA mutants was carried out at 37 °C for 3 h in 250-μL reaction mixtures that contained 25 μg of *Bst*NI-cleaved plasmid DNA, 4 mM each NTP, 16 mM GMP, 40 mM Tris-HCl, pH 8.1, 30 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT (dithiothreitol), 0.01% Triton X-100, 0.4 unit/μL RNasin (Promega), and 80 units/μL T7 RNA polymerase. The transcription products were phenol-extracted, ethanol-precipitated, and separated by a 12% PAGE/7 M urea gel. Full-length transcripts were eluted from gel slices by a Schleicher & Schuell electroelution apparatus, and ethanol-precipitated. The yield is typically 50–100 μg for a full-length transcript.

Aminoacylation with Cysteine. Aminoacylation was assayed at 37 °C in 24 μL of a buffer containing 20 mM KCl, 10 mM $MgCl_2$, 25 mM DTT, 2 mM ATP, 20 mM Tris-HCl (pH 7.5), 50 μM cysteine, 0.385 μM [^{35}S]cysteine (New England Nuclear, greater than 600 Ci/mmol), and 1–10 μM purified tRNA transcript [in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA] that was previously heated (70 °C, 2 min) and reannealed at room temperature (Hou *et al.*, 1993). Aminoacylation was initiated by adding 4 μL of an appropriately diluted purified *E. coli* cysteine–tRNA synthetase (e.g., 4 nM for the wild-type transcript) (Hou *et al.*, 1991). At various time points, aliquots of 4 μL of the aminoacylation reaction were removed to a 20-μL stop solution of 0.24 M iodoacetic acid/0.1 M sodium acetate (pH 5.0) in formamide, and the alkylation reaction was incubated at 37 °C for 30 min. A portion of the alkylation reaction (12 μL) was spotted onto a Whatman 3MM filter pad, precipitated with trichloroacetic acid, and washed as previously described (Schreier & Schimmel, 1972).

Chemical Modification with Dimethyl Sulfate (DMS). The tRNAs were labeled at the 3′ end with [5′-^{32}P]pCp (New England Nuclear) by T4 RNA ligase (New England Biolabs) at 4 °C overnight (Pan & Uhlenbech, 1992). The labeled tRNAs were purified by denaturing gel electrophoresis (12% PAGE/8 M urea), eluted by extraction of the gel slices with 0.125 M NH_4OAc, 0.125 mM EDTA, and 0.025% SDS, ethanol-precipitated, and counted. Modification with DMS was carried out with 4 μM tRNAs, 30 000 Cerenkov counts of the 3′-labeled tRNA in 10 mM $MgCl_2$/50 mM sodium cacodylate, pH 7.2, and 0.5% DMS (diluted in ethanol) for 5 min for the native condition. For the semi denaturing condition, tRNA was heated at 90 °C in water for 2 min, cooled on ice for 2 min, equilibrated in 1 mM EDTA and 50 mM sodium cacodylate, pH 7.2, and then treated with 0.5% DMS for 2 min. DMS resulted in chain scissions which, after aniline treatment (Peattie & Gilbert, 1980), generated labeled tRNA fragments that could be separated by gel electrophoresis and detected by autoradiography.

Chemical Modification with Kethoxal. Modification with kethoxal was carried out with 2.4 μM tRNAs in 50 mM sodium cacodylate, pH 7.2, and 0.02% kethoxal for 10 min at room temperature with 10 mM $MgCl_2$ for the native condition, and with 1 mM EDTA for the semidenaturing condition (previously heat–cool-treated as described for the DMS reaction) (Theobald *et al.*, 1988). Modifications were stopped by adding KOH–borate (pH 7.0) to a final concentration of 15 mM, followed by ethanol-precipitation. After modifications, tRNAs were denatured and annealed with a 5′-end-labeled oligonucleotide primer that was complementary to the 3′-end sequence of the tRNA from p61 to p76. The primer was extended by AMV reverse transcriptase (2 units, Life Sciences) in 6.3 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 40 mM KCl, 20 mM KOH–borate, and 0.25 mM each dNTP at 42 °C for 30 min. Primer extension stopped at the site of modification. The extended fragments were separated by denaturing gel electrophoresis and were detected by autoradiography (Theobald *et al.*, 1988; Wakao *et al.*, 1989; Ehresmann *et al.*, 1987).

RESULTS

E. coli $tRNA^{Cys}$ has a D3V4 configuration (three base pairs in the D stem and four nucleotides in the variable loop) rather than the D4V5 configuration that is observed in the cloverleaf structure for the majority of other tRNAs, including yeast $tRNA^{Phe}$. At position 13·22, where the D4V5 tRNAs have a complementary base pair, *E. coli* $tRNA^{Cys}$ has an A13·A22 mismatch. Adjacent to this mismatch is a U21 in the D loop, whereas more than 99% of all tRNAs have A21 or G21 (Sprinzl *et al.*, 1991). Inspection of available sequences of tRNAs shows that the coexistence of U21 and the D3V4 configuration is unique to *E. coli* $tRNA^{Cys}$, and is absent from all other tRNAs (Sprinzl *et al.*, 1991). Site-directed mutagenesis was performed in *E. coli* $tRNA^{Cys}$ to introduce substitution of A13 with U13 and of U21 with A21, and the addition of G47 to create a D4V5 configuration that carries an A21 (Figure 2). Individual nucleotide substitutions were also generated for comparison. Moreover, a U17 was added to the D4V5 configuration so that the size and shape of the D–V loops in *E. coli* $tRNA^{Cys}$ would resemble those in yeast $tRNA^{Phe}$. These nucleotide substitutions were tested for their ability to switch the base-pairing at G15·G48 to the Levitt configuration.

The switch was monitored by kethoxal reactivity at G48. Kethoxal can react with N1G and N2G to form a five-membered ring adduct (Figure 1) that can be detected during primer extension of the modified tRNA. The adduct was detected as the reverse transcriptase stopped the primer

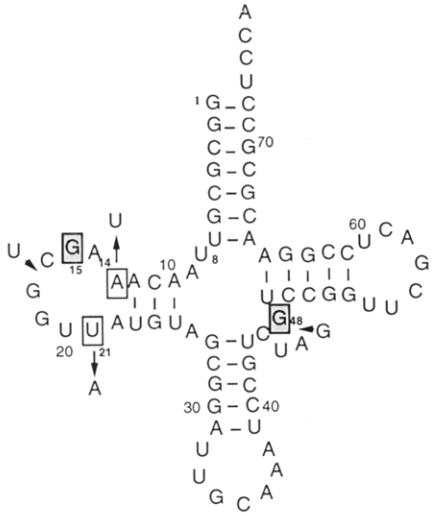

FIGURE 2: Nucleotide sequence and cloverleaf structure of *E. coli* $tRNA^{Cys}$. The G15 and G48 that establish an unusual tertiary interaction are indicated by shading. Nucleotide substitutions at A13 and U21 are indicated by open boxes, and insertion of G47 and U17 is shown by arrows.

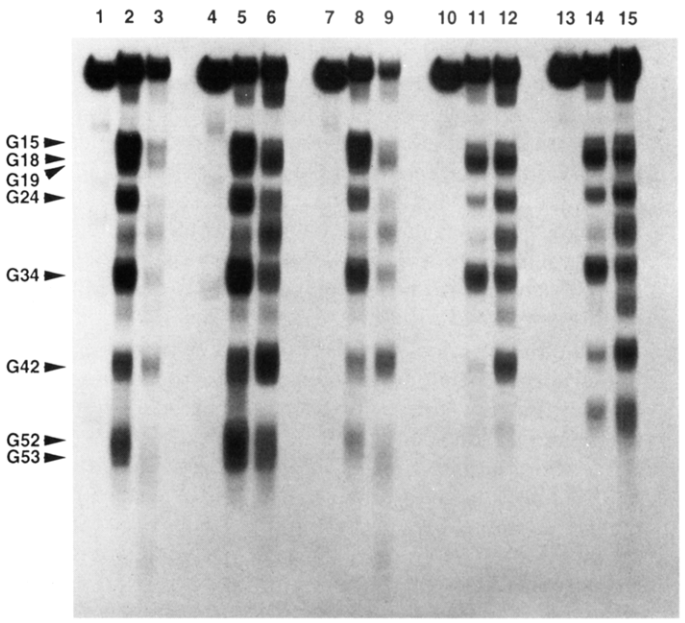

FIGURE 3: Phosphorimager analysis of the DMS reactivity at G15 in the wild type (lanes 1–3), U13 mutant (lanes 4–6), A21 mutant (lanes 7–9), U13A21 mutant (lanes 10–12), and U13A21/+G47 mutant (lanes 13–15) of *E. coli* $tRNA^{Cys}$. Lanes 1, 4, 7, 10, and 13 show controls, where individual tRNAs were not subjected to DMS treatment; lanes 2, 5, 8, 11, and 14 show the reactivity of individual tRNAs with DMS under native conditions; lanes 3, 6, 9, 12, and 15 show the reactivity of individual tRNAs with DMS under semidenaturing conditions. Arrows indicate the DMS cleavage products that end at G15, G18/G19, and G45, respectively.

extension on the tRNA template at the site of the kethoxal adduct. At G48 of the wild-type *E. coli* $tRNA^{Cys}$, kethoxal was reactive, indicating the presence of free N1G and N2G in the hydrogen-pairing scheme between G48 and G15 (Hou *et al.*, 1993). If the base-pairing had been converted to G15·G48 in a Levitt configuration, neither G48 nor G15 would have a free N1G, and thus neither would be reactive with kethoxal. Introduction of U13 or A21 alone had no effect on the kethoxal reactivity at G48 (Table 1). However, the combination of U13 and A21 substitutions reduced the kethoxal reactivity at G48 to a marginal level, indistinguishable from a control which did not react with kethoxal. It should be noted that the primer extension reaction had nonspecific stops at G48 due to random pauses by the reverse transcriptase (gels not shown), and therefore the reactivity of kethoxal at G48 was measured relative to the control. Further addition of G47 and U17 eliminated residual kethoxal reactivity at G48.

The elimination of kethoxal reactivity at G48 suggests that the N1 of G48 was involved in hydrogen-pairing with G15. If this base-pairing is maintained by sugar–phosphate backbones that mimic those in the Levitt base pair at positions 15 and 48, then the purine ring of G15 would stack with the purine ring of A14 such that the N7 of G15 would be protected from the chemical probe DMS. The reactivity with DMS at G15 was therefore determined for the mutants. The DMS reaction resulted in scission in the polynucleotide chain at G15, which could be detected as a truncated fragment on a denaturing polyacrylamide gel. The tRNA was end-labeled for the DMS reaction, and the intensity of the radioactivity for the DMS cleavage product at G15 was subjected to analysis by phosphorimager (Figure 3). The intensity was normalized to an internal control at G34 that also reacted with DMS. Introduction of U13, or A21, or the addition of G47 had no substantial effect on the reactivity with DMS at G15 (Table 1). The double substitution U13A21 markedly decreased the ability of G15 to react with DMS to only 5% of that of the wild type. The 20-fold decrease in the DMS reactivity at G15 would be consistent with an increased stacking interaction between G15 and A14. While other possibilities for a decrease in DMS reactivity at G15 exist, an increase in the stacking interaction would provide evidence for the switch of base-pairing at G15·G48 to a Levitt configuration. Subsequent addition of G47 and U17 did not enhance the stacking interaction further, and the mutant retained the 5% DMS reactivity at G15 as exhibited by the U13A21 mutant. Under

Table 1: Nucleotide Substitutions That Lead to Conversion of the Tertiary Base-Pairing at G15·G48 in *E. coli* $tRNA^{Cys}$

nucleotide substitutions	aminoacylation relative k_{cat}/K_m[a]	kethoxal reactivity at G48[b]	DMS reactivity at G15[c]
wild type	1.0	>1.0	1.0
A13 → U13	0.33	>1.0	0.73
U21 → A21	0.22	>1.0	0.67
addition of G47	0.81	nd[d]	1.0
A13 → U13 and U21 → A21	0.0067	1.0	0.05
A13 → U13, U21 → A21 and addition of G47	0.0069	0.41	0.05
A13 → U13, U21 → A21, and addition of G47 and U17	0.0064	<0.10	<0.05

[a] The initial rate of aminoacylation was measured with 4 nM cysteine–tRNA synthetase [specific activity, 1.8 nmol min^{-1} (μg of protein)$^{-1}$ and tRNA concentrations that ranged from 1 to 16 μM. The K_m of the wild-type tRNA transcript for the enzyme is 5 μM. The k_{cat}/K_m was obtained from the slope of the initial rates vs tRNA concentrations and had an accuracy of 10%. [b] The kethoxal reactivity at G48 was indicated as a relative ratio to the reactivity of G48 in the control that did not react with kethoxal. The ">1.0" denotes a greater reactivity than the control whereas a ratio that is equal to or smaller than 1.0 means no reactivity. [c] The DMS reactivity at G15 was measured by the phosphorimager to estimate the intensity of the reaction, which was then normalized to an internal control at G34. The reactivity is then given as a ratio to the wild type. [d] Not determined

the same reaction conditions, yeast tRNA^{Phe} showed no reactivity with DMS at G15 (not shown).

The residual 5% DMS reactivity at G15 in the U13A21 double mutant suggests some variation from the normal Levitt base pair. It is not known whether this variation reflects the intrinsic difference between a G·G base pair and a G·C base pair in the Levitt configuration. It should be noted that, by using Pb^{2+} cleavage of tRNA as an indicator for the overall structural integrity, a G15·G48 variant of yeast tRNA^{Phe} had a 5-fold reduced rate of cleavage compared to that of the wild type (Behlen *et al.*, 1990).

While the switch of G15·G48 to the Levitt configuration in the U13A21 mutant was supported by the kethoxal and DMS reactivities, it was also accompanied by a decrease in the aminoacylation activity with cysteine. The catalytic efficiency of aminoacylation for the cysteine enzyme (k_{cat}/K_m) was investigated with a series of tRNA concentrations that were below the K_m of the mutant. Under this condition, the initial rate was proportional to k_{cat}/K_m and was used to compare the catalytic efficiency of the mutant relative to the wild type. The U13A21 mutant and the mutants with these two substitutions and the addition of G47 and U17 all showed a more than 100-fold reduction in k_{cat}/K_m (Table 1). This decrease in k_{cat}/K_m is in the same range as the decrease of k_{cat}/K_m due to alteration of G15·G48 in the wild-type tRNA^{Cys} to either G15·G48 or C15·G48. The extents of reduction in aminoacylation among these three mutants were essentially the same. On the other hand, single substitution of U13 or A21 had a less than 2-fold effect on k_{cat}/K_m. The results highlighted the importance of the U13A21 double substitution in catalysis. Because catalysis is concomitant with tRNA conformational change, the lack of aminoacylation catalysis in the double mutant suggested a structural defect. Thus, the results of aminoacylation were consistent with the results of kethoxal and DMS chemical probes that showed a structural switch at G15·G48 upon introduction of U13 and A21.

DISCUSSION

The tRNAs investigated in this study are run-off transcripts that contain no modified bases. The native *E. coli* tRNA^{Cys} has modifications at positions 8, 20, 21, 32, 37, 39, 54, and 55. Previous study has shown that the native tRNA reacted to DMS and kethoxal in the same manner as the wild-type transcript, and has only a 5-fold higher catalytic efficiency of aminoacylation (Hou *et al.*, 1993). All the mutants that affect the G15·G48 base pair have a more than 100-fold decrease in catlaytic aminoacylation. Thus, it appears that although the modified bases are believed to contribute to the stability of overall tRNA tertiary structure (Rich & RajBhandary, 1976), they do not have a major role in the formation of the G15·G48 base pair.

Comparison of the sequence of *E. coli* tRNA^{Cys} with all other tRNAs suggests that there are perturbations of the stacking interactions in the D stem and loop of this tRNA (see below). The perturbation is best demonstrated by the reactivity of G15 to DMS in the wild-type tRNA, whereas purine 15 is normally unaccessible to chemical probes in other tRNAs. Introduction of U13 and A21 shifts the accessible G15. One likely consequence of this shift is that G15 would stack with A14. On the basis of the DMS reaction, the level of stacking would be within 95% of that in the yeast tRNA^{Phe}. The U13A21 mutant has a D4V4 configuration, which is one nucleotide short of the D4V5 configuration of yeast tRNA^{Phe} in the variable loop. Addition of G47 in the variable loop does not further enhance the stacking interaction between

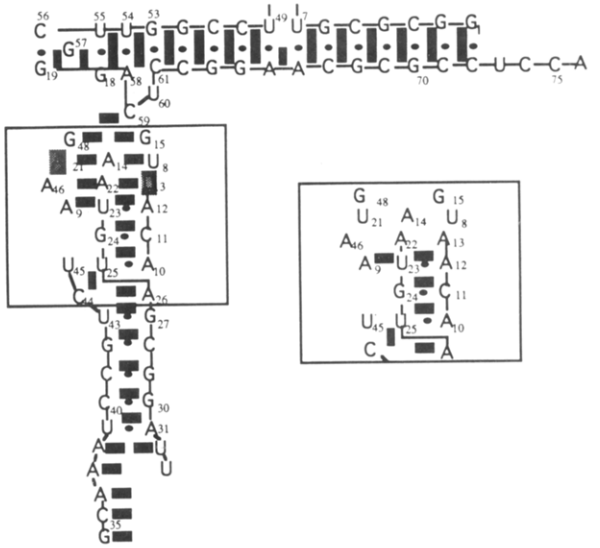

FIGURE 4: Proposed stacking interactions between various nucleotides in the U13A21 mutant of *E. coli* tRNA^{Cys}, where nucleotides U13 and A21 are shaded. The region of the D stem–loop that differs in stacking interactions between the mutant and the wild type is indicated by a box. The proposed perturbation of the stacking interactions in the wild type is shown in the insert. The stacking interactions that are predicted to preserve as in the analogous positions in the crystal structure of yeast tRNA^{Phe} are shown by black bars. Only nucleotides that are involved in the stacking interactions are shown. The connectivity of the nucleotide sequence is indicated by the number.

G15 and A14. In the crystal structure of yeast tRNA^{Phe}, nucleotide 47 is not involved in the tertiary hydrogen or stacking interactions, and is projected away from the center of the tRNA. If the reduced accessibility at G15 reflects enhanced stacking interaction, it is likely that the U13A21 mutant of *E. coli* tRNA^{Cys} has acquired most, or all, of the stacking interactions in the D stem–loop that are observed in yeast tRNA^{Phe}.

Figure 4 shows the proposed stacking interactions in *E. coli* tRNA^{Cys} and in the U13A21 mutant. The figure is drawn on the basis of the stacking interactions in yeast tRNA^{Phe}, but with substitution of the nucleotide sequence for *E. coli* tRNA^{Cys}. The U13A21 mutant shares the composition of A21, U8, and A14 with yeast tRNA^{Phe} and therefore can be proposed to establish a hydrogen bond between the N1 of A21 and the 2′-OH of ribose 8 as is observed in yeast tRNA^{Phe} (Kim *et al.*, 1974a,b; Robertus *et al.*, 1974). The A21·U8·A14 tertiary interaction in yeast tRNA^{Phe} forms a plane, which enables A21 to stack under the Levitt base pair (Kim *et al.*, 1974b; Ladner et al., 1975). If the proposed model is correct, A21 in the U13A21 mutant would also stack under the Levitt-like G15·G48. Below this A21·U8·A14 tertiary interaction in yeast tRNA^{Phe} is a C13·G22 base pair that forms two hydrogen bonds in the major groove between G22 and a positively charged m^{7}G46 (Kim *et al.*, 1974a,b; Robertus *et al.*, 1974). This C13·G22·G46 tertiary interaction contributes further to the stacking interactions in the D stem. A similar tertiary interaction of U13·A22·A46 can be proposed for the U13A21 mutant, whereby the N1 and N6 of A46 would form a hydrogen bond, respectively, with the N6 and N7 of A22 (Kim *et al.*, 1974b). In fact, the combination of A46 and U13·A22 is found in the sequence of yeast tRNA^{Arg}, which also has A21 and U8·A14 (Sprinzl *et al.*, 1991). This suggests that the proposed hydrogen and stacking interactions in the U13A21 mutant are represented in a naturally existing tRNA.

In contrast, the wild-type tRNA^{Cys} contains a U21, which is modified as dihydrouridine (D) in the native tRNA.

Although U21 (or D21) in principle can form a hydrogen bond with the 2′-OH of ribose 8, it is not known if this hydrogen bond would maintain U21 on the same plane of U8·A14. Considerable variation in the ribose phosphate backbone at U21 can be envisaged that can interfere with the stacking interaction between U8·A14 and G15·G48. Conversely, the mismatched A13·A22 does not mean that nucleotides 22 and 46 cannot be stacked. All of the D3V4 tRNAs have mismatched nucleotides at 13 and 22, yet they maintain a Levitt base pair. For example, the crystal structure of *E. coli* $tRNA^{Gln}$ shows that the mismatched A13·A22, which pairs with U46, is stacked under A21·U8·A14 while maintaining a normal Levitt G15·C48 base pair (Rould *et al.*, 1989). It is possible that the bulkier A at positions 13, 22, and 46 cannot be easily accommodated in the same plane. Introduction of a complementary U13 may remove some of the constraints and enhance the stacking interaction at 13·22·46. Thus, installation of both A21·U8·A14 and a U13·A22 base pair that could stabilize the stacking interaction at 13·22·46 is necessary to maintain a Levitt-like G15·G48 base pair.

Examination of the DMS chemical probe data (Figure 3) suggests that, except for the switch at G15·G48, all tRNA mutants that were investigated here maintain an overall tertiary structure similar to that of the wild type. This is indicated by their relative reactivities to DMS at various positions. For example, phosphorimager analysis of band intensity shows that G24 in the wild type reacts to DMS almost twice as strongly as G42. This ratio of 2 for G24 vs G42 is preserved in all four mutants under the native conditions. However, it is fair to say that the U13A21 and U13A21/+G47 mutants may have acquired additional structural changes from the wild type at positions outside of G15·G48. These changes are reflected by the enhanced DMS reactivities at G24 and G34 under semidenaturing conditions. Because these changes are not as significant as the changes at G15 for these mutants, they are probably minor. It is possible that the additional minor changes are brought about by the switch at G15·G48.

Inspection of tRNA sequences shows that, besides *E. coli* $tRNA^{Cys}$, only three other tRNAs have a pyrimidine at position 21. All these tRNAs have an unusual nucleotide composition at positions 15 and 48; e.g., U15·A48 for *Aedes albopictus* $tRNA^{Gly}$ that has U21, A15·A48 for yeast $tRNA^{Pro}$ that has C21, and U15·C48 for a bovine $tRNA^{Ser}$ that has U21 (Sprinzl *et al.*, 1991). Although the detailed structure of these tRNAs is not known, the presence of pyrimidine 21 appears to promote variations from the normal Levitt base pair. Thus, the use of G15·G48 to elucidate U8·A14·A21 as one of the structural elements that contribute to the Levitt base pair has useful applications. It will be interesting to establish the structural requirements in a tRNA that contribute to other tertiary interactions. The understanding of these requirements would ultimately assist in our ability to design and predict RNA tertiary structure.

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