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Transfer RNA aminoacylation: Identification of a critical ribose 2′-hydroxyl–base interaction

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ABSTRACT

To understand the relationship between tRNA architecture and specific aminoacylation by aminoacyl-tRNA synthetases, we performed kinetic assays of *Escherichia coli* tRNA^{Pro} molecules containing single deoxynucleotide substitutions. We identified an important 2′-hydroxyl group at position U8 (of 22 positions probed). Chemical modification studies showed that this 2′-hydroxyl interacts with either the N1 or the exocyclic amine of G46 in a hydrogen bonding interaction that contributes 1.8 kcal/mol to the free energy of activation for aminoacylation. Molecular modeling of tRNA^{Pro} supports the existence of this interaction. This is the first study to identify a specific ribose 2′-hydroxyl–base interaction in the core region of a tRNA molecule that makes a thermodynamically significant contribution to aminoacylation.

Keywords: chemical modification; chemical RNA synthesis; proline-tRNA synthetase; semi-synthetic tRNAs; tertiary interactions; tRNA charging

INTRODUCTION

Recent studies have elucidated tertiary interactions in the *Tetrahymena* group I intron (Pyle et al., 1992) and in the hammerhead ribozyme (Pley et al., 1994) that highlight the important role of 2′-hydroxyl–base interactions in both protein–RNA and RNA–RNA recognition events. The importance of 2′-hydroxyl groups in tRNA structure has also been established (Kim et al., 1974a; Ladner et al., 1975; Holbrook et al., 1978; Romby et al., 1987). However, few studies have examined directly the role that 2′-hydroxyls play in aminoacylation of tRNAs by aminoacyl-tRNA synthetases. These enzymes catalyze the attachment of amino acids to specific or cognate tRNAs and must discriminate against noncognate tRNAs. Synthetic RNA duplexes that mimic the acceptor stem of tRNA^{Ala} are efficient substrates for *Escherichia coli* alanine-tRNA synthetase (AlaRS) (Musier-Forsyth et al., 1991) and were used previously to examine the role of 2′-hydroxyls in specific aminoacylation by AlaRS (Musier-Forsyth & Schimmel, 1992). Single deoxynucleotide substitutions were incorporated into duplex^{Ala} variants and used to identify an important cluster of minor groove 2′-hydroxyl functional contacts near a critical guanosine 2-amino group.

We have previously described a method for incorporating single deoxynucleotides into full-length tRNAs prepared from two fragment molecules (Liu & Musier-Forsyth, 1994). This allows us to measure the individual contributions of 2′-hydroxyl groups toward lowering the free energy of activation for aminoacylation of tRNAs. In previous work, nine individual 2′-hydroxyls located in either the acceptor stem (at positions 2, 3, 5, and 7) or the D stem-loop (at positions 10, 11, 12, 13, and 15) of *E. coli* tRNA^{Pro} were tested for their role in aminoacylation by *E. coli* proline-tRNA synthetase (ProRS) (Liu & Musier-Forsyth, 1994). None of these single deoxynucleotide substitutions resulted in significant decreases in aminoacylation efficiency. In the present work, we have probed additional 2′-hydroxyls in the acceptor stem, D-stem, and TΨC stem-loop domains of semi-synthetic tRNA^{Pro} variants. Only minor decreases in aminoacylation efficiency are observed upon 2′-hydroxyl → hydrogen substitutions at all but one position. A 22-fold decrease in the aminoacylation efficiency was measured upon incorporation of a single deoxynucleotide at position U8. Chemical modification studies and molecular modeling support the existence of a tertiary interaction between the critical 2′-hydroxyl

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at U8 and a guanosine located at position 46 in the variable loop domain of $tRNA^{Pro}$. This study suggests that specific 2′-hydroxyl–base interactions may be used as recognition elements by aminoacyl-tRNA synthetases to discriminate cognate from noncognate substrates.

RESULTS AND DISCUSSION

Two schemes used to prepare semi-synthetic tRNAs are shown in Figure 1. Briefly, a chemically synthesized 5′ (path a) or 3′ (path b) oligonucleotide (typically 15 or 16 nt long) is heat-annealed to the corresponding in vitro transcribed 3′- or 5′-3/4 tRNA fragment. We have previously shown that D-loop nucleotides 16–17a (Liu & Musier-Forsyth, 1994), and TΨC-loop nucleotides 58–60 (L.-P. Yap & K. Musier-Forsyth, in prep.) are dispensable for aminoacylation of $tRNA^{Pro}$, and these are not included in our semi-synthetic constructs (Fig. 1, brackets). A total of 22 single deoxynucleotide substitutions located in the acceptor stem and D and TΨC

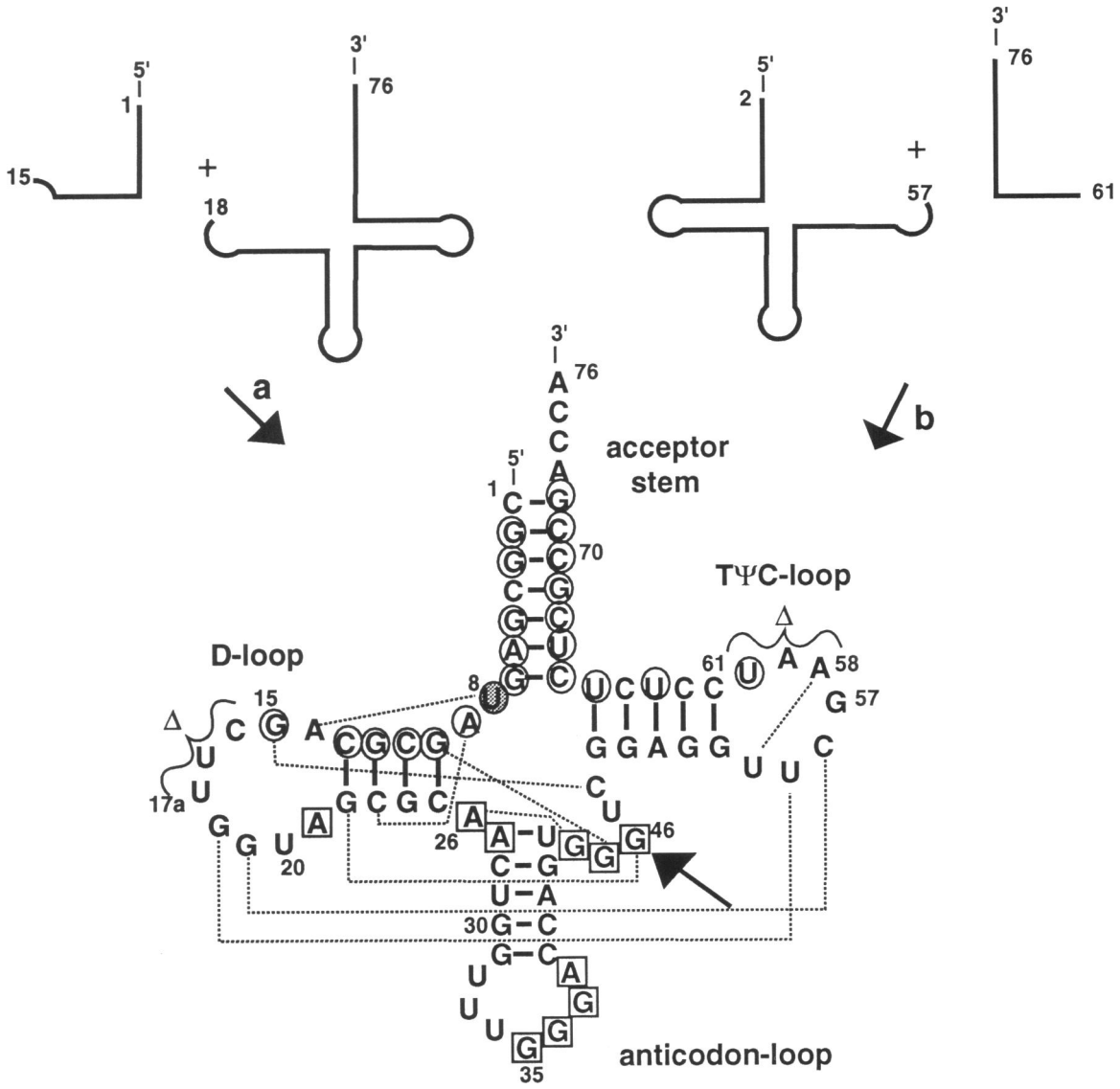

FIGURE 1. Structure of the UGG isoacceptor of *E. coli* $tRNA^{Pro}$ (unmodified) used in these studies. Transfer RNAs were prepared from two fragment molecules (Liu & Musier-Forsyth, 1994) and contained a break in the phosphodiester backbone either in the D-loop (path a) or the TΨC-loop (path b). In each construct, three nucleotides could be deleted (16–17a and 58–60, bracketed, △) without affecting aminoacylation efficiency (Liu & Musier-Forsyth, 1994; L.-P. Yap & K. Musier-Forsyth, in prep.). Shorter fragments (5′-15-mers and 3′-16-mers) were chemically synthesized and annealed to longer fragments prepared by in vitro transcription. Dotted lines represent proposed tertiary interactions and are based on the known structure of yeast $tRNA^{Phe}$ (Kim et al., 1974b). Circled nucleotides indicate the location of 22 single deoxynucleotide substitutions that have been incorporated into the annealed tRNAs. The substitution of dU at position 8 (shaded) was the only change that had a significant effect on aminoacylation efficiency (see Table 1). Nucleotides that were probed in chemical modification studies with DMS (A residues) and kethoxal (G residues) are indicated by open squares. The solid arrow points to the only nucleotide that consistently displayed increased modification with kethoxal in the dU8 variant (see Fig. 4).

stem-loop regions have now been incorporated into the chemically synthesized fragment of semi-synthetic $tRNA^{Pro}$ molecules (Fig. 1, circled nucleotides). The results of aminoacylation assays show that removal of the 2′-hydroxyl group at the majority of positions tested has a relatively minor effect on aminoacylation (Liu & Musier-Forsyth, 1994) (Table 1). Strikingly, a major reduction in aminoacylation efficiency (22-fold) was seen upon deoxynucleotide substitution at only one position, U8 (Fig. 1, shaded; Table 1). Figure 2 shows a comparison of the initial rate of aminoacylation with proline of the all-ribo versus the dU8 variant $tRNA^{Pro}$. The difference in the free energy of activation for aminoacylation between the all-ribo and the dU8 substrate, $-\Delta\Delta G^{0\ddagger}$, is 1.8 kcal/mol (Table 1).

In order to assess whether the global folding of the dU8 variant is disrupted, native PAGE was used as described previously (Liu & Musier-Forsyth, 1994). The 3′-3/4 tRNA apparently has multiple conformations and runs as four major bands on a native gel (Fig. 3, lane 3). The annealed dU8-containing tRNA, on the other hand, co-migrates with the all-ribo annealed tRNA and a full-length $tRNA^{Pro}$ transcript (Fig. 3,

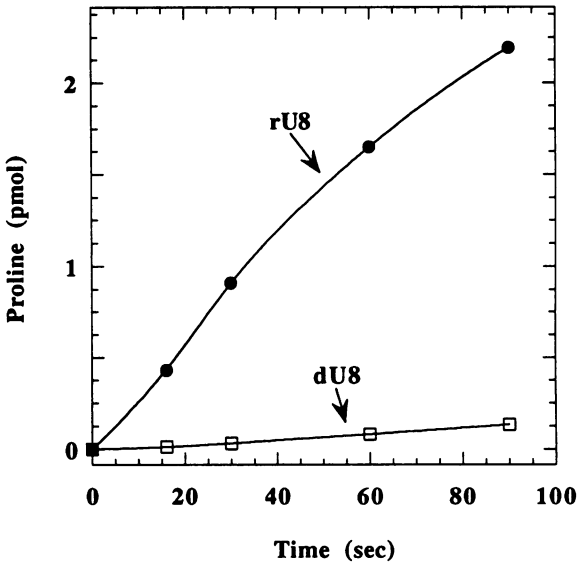

FIGURE 2. Aminoacylation of semi-synthetic $tRNA^{Pro}$ molecules with proline. The assays were carried out as described in the Materials and methods, using 4 μM annealed substrate and 0.1 μM ProRS. Each time point represents the incorporation of proline per 15-μL reaction aliquot. The curve labeled rU8 corresponds to the all-ribo 5′-15-mer annealed to the 3′-59-mer. The curve labeled dU8 corresponds to the 5′-dU8-15-mer annealed to the 3′-59-mer.

TABLE 1. The effect of single deoxynucleotide substitutions on prolylation of annealed $tRNA^{Pro}$ molecules.

Synthetic oligonucleotide	k_{cat}/K_m (relative)[a]	Change in specificity (x-fold)	$-\Delta\Delta G^{0\ddagger}$ (kcal/mol)
5′-15-mer[b]			
All-RNA	1.0	1.0	0
dA6	1.8	1.8	−0.35
dU8	0.046	−22	1.8
dA9	1.8	1.8	−0.35
3′-16-mer[c]			
All-RNA	1.0	1.0	0
dU60[d]	0.51	−2.0	0.40
dU63	0.46	−2.2	0.46
dU65	0.77	−1.3	0.15
dC66	2.0	2.0	−0.41
dU67	0.52	−1.9	0.39
dC68	1.0	1.0	0
dG69	0.97	−1.0	0.018
dC70	2.5	2.5	−0.55
dC71	1.2	1.2	−0.11
dG72	1.2	1.2	−0.11

[a] The k_{cat}/K_m of single deoxynucleotide-substituted annealed tRNAs is reported relative to the corresponding all-RNA annealed tRNA, which was assigned a value of 1.0. Under the experimental conditions employed, initial rates of aminoacylation were proportional to substrate concentration. $\Delta\Delta G^{0\ddagger}$ is given by $RT \ln(k_{cat}/K_m)^{\text{deoxy variant}}/(k_{cat}/K_m)^{\text{wild type}}$, where "wild type" refers to the corresponding all-RNA annealed tRNA.

[b] This set of assays was performed by annealing a 5′-15-mer to a 3′-59-mer. The k_{cat}/K_m of the all-ribo 5′-15-mer annealed to the 3′-59-mer is 0.020 $\mu M^{-1} s^{-1}$. The values reported are averages of at least five determinations, using tRNA concentrations ranging from 1 to 4 μM, with an estimated error of ±17%.

[c] This set of assays was performed by annealing 3′-16-mers to a 5′-ΔC1-57-mer. The k_{cat}/K_m of the all-ribo 3′-16-mer annealed to the 5′-ΔC1-57-mer is 0.027 $\mu M^{-1} s^{-1}$. The values reported are averages of at least three determinations, using tRNA concentrations ranging from 1 to 8 μM, with an estimated error of ±19%.

[d] This oligonucleotide was prepared as a 17-mer, and results are relative to an all-RNA 17-mer.

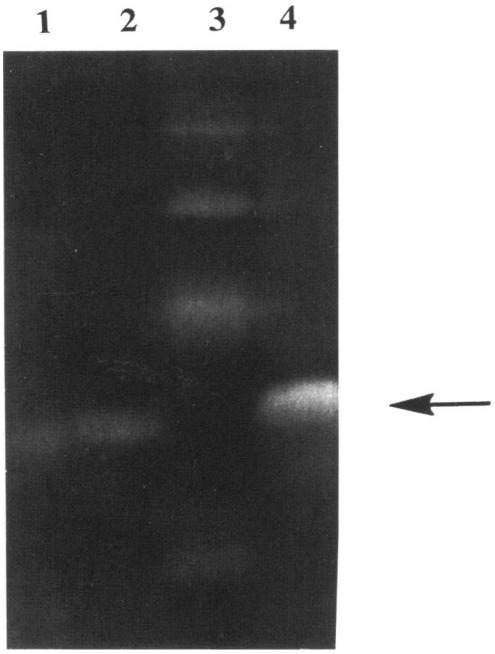

FIGURE 3. Ethidium bromide-stained, native 12% polyacrylamide gel. Lane 1, annealed 3′-59-mer + all-ribo 5′-15-mer; lane 2, 3′-59-mer + 5′-dU8-15-mer; lane 3, 3′-59-mer alone; lane 4, full-length in vitro-transcribed ΔC1-$tRNA^{Pro}$. Arrow points to the position of full-length $tRNA^{Pro}$. Native gels were prepared and run as described previously (Liu & Musier-Forsyth, 1994).

lanes 1, 2, 4). Therefore, the reduction in aminoacylation efficiency observed with the dU8 variant is not caused by a change in the global tRNA structure.

Although native gel electrophoresis is a good indicator of global architecture, it does not provide information about subtle structural differences that may exist between annealed tRNA variants. The crystal structure of yeast $tRNA^{Phe}$ suggests that the N1 of A21 makes a hydrogen bond to the ribose of U8 (Kim et al., 1974b; Ladner et al., 1975). Seven of the nine tertiary interactions that contribute to the folded structure of $tRNA^{Phe}$ (Kim et al., 1974b; Robertus et al., 1974; Sampson et al., 1990) are identical in $tRNA^{Pro}$ (Fig. 1). Therefore, the crystal structure of yeast $tRNA^{Phe}$ is likely to be a good model for predicting interactions in the core region of $tRNA^{Pro}$. We decided to use chemical modification as a direct probe of core interactions that might be affected by deletion of the 2′-hydroxyl group of U8. Figure 4A shows the results of primer extension experiments performed following dimethyl sulfate (DMS) treatment of annealed tRNAs. DMS modification of the N1 position of adenine residues interferes with reverse transcription and results in primer extension stops (Inoue & Cech, 1985). Weak stops in reverse transcription are seen at A21 and A27, whereas strong stops are seen at A26 and A38 in both the all-RNA and the dU8 variant. These results show that N1 of A21 does not become significantly more accessible upon removal of the 2′-hydroxyl of U8. The extensive network of hydrogen bonding and base–base stacking interactions in the core region of the tRNA is likely to block access to this part of the molecule (Romby et al., 1987). This result, therefore, does not rule out the possibility of a direct contact between the ribose 2′-hydroxyl of U8 and the N1 of A21.

We next decided to probe the annealed tRNAs with kethoxal, a reagent that modifies the N1 and N2 positions of guanine bases (Litt & Hancock, 1967). Primer extension experiments show moderate to strong stops at positions G35, G36, and G37 of both the all-ribo and the dU8 tRNAs (Fig. 4B). Differential reactivity, however, is seen in the variable loop bases G45 and G46, whereas G44 shows little reactivity above the background signal (−) in either case. In this particular experiment, the reactivity of the all-ribo substrate is reduced to 29% of the dU8 variant at G45. A more dramatic effect, however, is observed at position 46 (Fig. 1, arrow). In the experiment shown in Figure 4B, the all-ribo variant failed to react with kethoxal, whereas the dU8 variant displayed significant reactivity at this position. To confirm these results, two other independent modification/primer extension experiments were per-

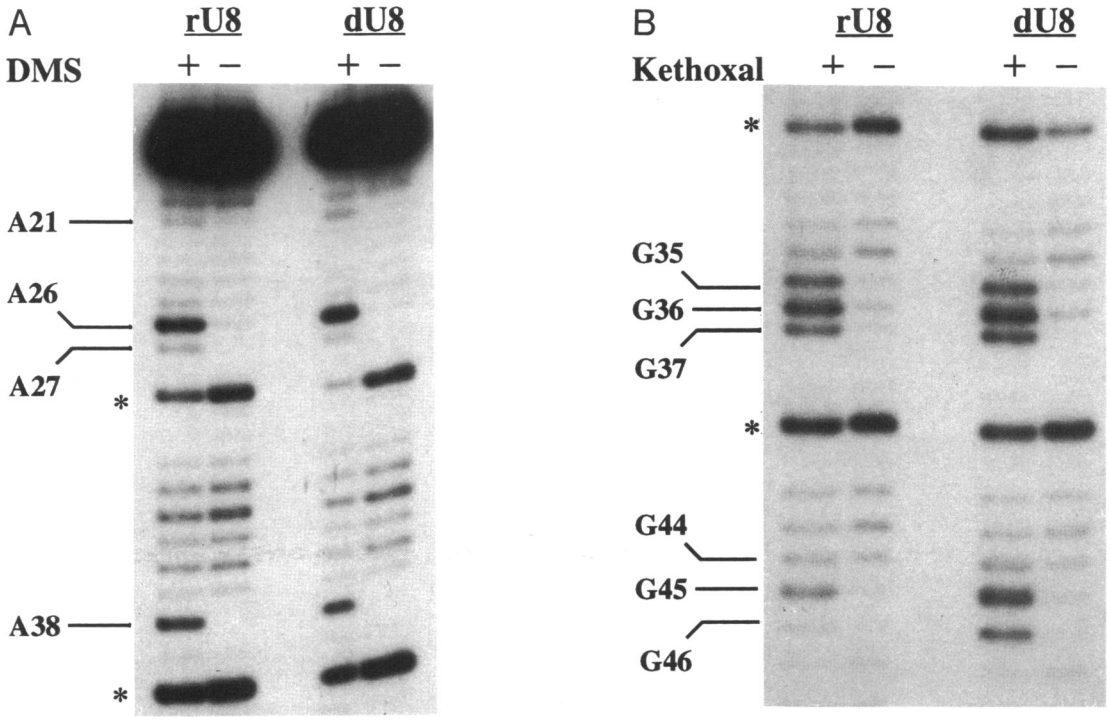

FIGURE 4. Results of chemical probing experiments using wild-type (rU8) and mutant (dU8) annealed tRNAs. **A:** Autoradiogram showing reverse transcription products of DMS-modified (+) and unmodified (−) annealed $tRNA^{Pro}$ molecules. Nucleotides numbered on the left indicate positions of A residues that were accessible to DMS attack. Positions were identified using a sequencing ladder (not shown). **B:** Autoradiogram showing reverse transcription products of kethoxal-modified (+) and unmodified (−) annealed $tRNA^{Pro}$ molecules. Positions of G residues in $tRNA^{Pro}$ that are susceptible to kethoxal modification are numbered on the left. Asterisks indicate strong DMS- or kethoxal-independent reverse transcriptase pause sites. Results were quantitated by densitometry (not shown).

formed. In these additional experiments (not shown), no difference in reactivity was observed at position G45, whereas significantly increased kethoxal modification was consistently observed in the dU8 variant at G46. Thus, in the absence of a 2′-hydroxyl at position U8, G46 becomes kethoxal-accessible. This suggests that N1 and N2 of G46 are free in the dU8 variant, whereas they may form a hydrogen bond with the 2′-hydroxyl of U8 in the all-ribo wild-type tRNA. We cannot rule out the possibility that the dramatic change in reactivity at position 46 is an indirect result of a change in tertiary structure elsewhere in the tRNA. For example, the disruption of an interaction with the N1 of A21 (which our DMS modification experiments failed to detect) may increase kethoxal reactivity at G46. However, using coordinates from the known X-ray crystal structure of yeast $tRNA^{Phe}$ (Kim et al., 1974a; Ladner et al., 1975), molecular modeling of *E. coli* $tRNA^{Pro}$ (Fig. 5) indicates that the ribose 2′-hydroxyl of U8 and the amino group of G46 are within hydrogen bonding distance (2.6 Å). The N1 of G46 is 4.6 Å removed from the 2′-hydroxyl of U8, and distances from the N1 and N2 positions of G44 and G45 to the 2′-hydroxyl of U8 are all >10 Å. These distances are outside the range of typical hydrogen bonding interactions in nucleic acids (Saenger, 1983). Therefore, our experimental data (Figs. 1, 4) and molecular modeling (Fig. 5) support the existence of a direct interaction between the 2′-hydroxyl of U8 and the amino group of G46. It should be noted that U8 is conserved in almost all non-mitochondrial elongator tRNAs and G46 is present in 82% of these tRNAs (Sampson et al., 1990; Steinberg et al., 1993). In the structure of yeast $tRNA^{Phe}$, m^7G46 is involved in a triple interaction with the G22:C13 pair of the D-stem (Kim et al., 1974a). In particular, N2 and N1 of m^7G46 are hydrogen bonded to O6 and N7 of G22. Modeling of $tRNA^{Pro}$ is consistent with the proposal that an additional hydrogen bond exists between the amino group of G46 and the 2′-hydroxyl of U8. The existence and general importance of this 2′-hydroxyl–base interaction for recognition and aminoacylation of tRNAs by aminoacyl-tRNA synthetases other than *E. coli* ProRS remains to be investigated. These results, nevertheless, suggest that intramolecular 2′-hydroxyl tertiary contacts may be used by synthetases to assist in discriminating cognate from noncognate tRNAs.

Of the 13 new 2′-hydroxyl groups probed in this work, 11 are solvent-exposed and are potential sites of interaction with ProRS. These positions, however, are not critical for aminoacylation. Of the remaining two groups tested (the 2′-hydroxyls of U8 and A9), only the 2′-hydroxyl of U8 was shown to be critical for aminoacylation. In yeast $tRNA^{Phe}$, the 2′-hydroxyl of A9 interacts with the N4 of C11 (Holbrook et al., 1978). Although a similar interaction is expected in *E. coli* $tRNA^{Pro}$, ProRS is not sensitive to loss of this interaction. Therefore, this enzyme appears to be differentially sensitive to the loss of the ribose–base interaction involving the 2′-hydroxyl of U8, but not the A9–C11 interaction.

Previous experiments showed that 2′-hydroxyl groups are important in substrate recognition by AlaRS (Musier-Forsyth & Schimmel, 1992) and in RNA–RNA

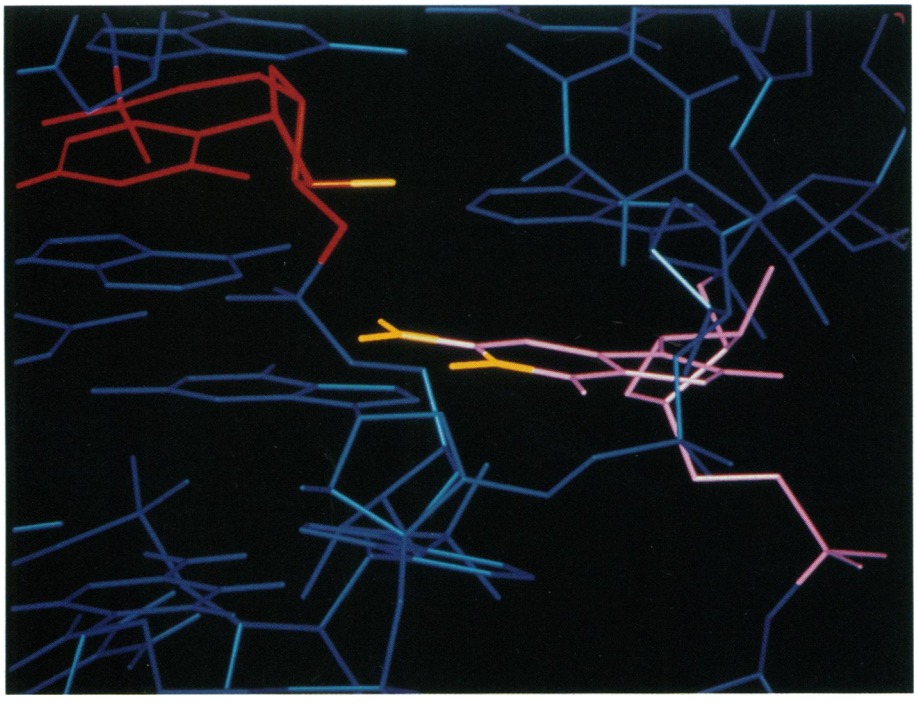

FIGURE 5. Model showing nucleotides in the core region of *E. coli* $tRNA^{Pro}$. Coordinates for $tRNA^{Phe}$ were employed to generate this model using Insight II (Biosym Technologies) on an IRIS Indigo XS24 workstation (Silicon Graphics, Inc.). G46 is shown in pink and U8 is in red. Atomic groups proposed to be involved in the tertiary interaction identified in this study (the 2′-hydroxyl of U8 and the N1 or 2-amino group of G46) are in gold.

recognition events leading up to splicing of the *Tetrahymena* group I intron P1 helix (Pyle & Cech, 1991; Strobel & Cech, 1995). In the latter system, evidence for an interaction between the N1 of A302 in the catalytic core of the ribozyme and one of the important substrate 2′-hydroxyls has been obtained (Pyle et al., 1992). Biochemical data on modification of 2′-hydroxyl functional groups that result in decreased hammerhead ribozyme activity correlate well with interactions observed in the recent X-ray crystallographic structure of a hammerhead RNA–DNA ribozyme inhibitor complex (Pley et al., 1994). A ribose tertiary interaction involving U8 was previously observed in the crystal structure of $tRNA^{Phe}$ (Kim et al., 1974b; Ladner et al., 1975). Our results demonstrate that a similar contact in $tRNA^{Pro}$ is energetically important for aminoacylation. In particular, we show that a specific 2′-hydroxyl–base interaction in the core of a tRNA molecule makes a thermodynamically significant contribution to RNA folding and to aminoacylation by an aminoacyl-tRNA synthetase. These results, along with previous data (Pyle et al., 1992; Pley et al., 1994), demonstrate that specific 2′-hydroxyl–base interactions that are likely to be involved in the maintenance of proper RNA architecture are important in both protein–RNA and RNA–RNA recognition events.

MATERIALS AND METHODS

Protein preparation

The procedure used to prepare ProRS has been described (Liu & Musier-Forsyth, 1994). ProRS protein concentrations were based on active-site titrations using the adenylate burst assay (Fersht et al., 1975). T7 RNA polymerase was purified according to Grodberg and Dunn (1988) from *E. coli* strain BL-21/pAR 1219, which was a gift of F. William Studier.

RNA preparation

Synthetic RNA and mixed RNA–DNA oligonucleotides were prepared by chemical synthesis on a Gene Assembler Plus (Pharmacia) using the phosphoramidite method (Scaringe et al., 1990). Oligonucleotides were purified on 16% polyacrylamide gels.

*Bst*NI linearized plasmid DNA encoding the $tRNA^{Pro}$ gene in front of a T7 RNA polymerase promoter was used to prepare in vitro run-off full-length ΔC1-$tRNA^{Pro}$ as described previously (Liu & Musier-Forsyth, 1994). The 3′-3/4 $tRNA^{Pro}$ transcript (59-mer) was prepared in a similar fashion (Liu & Musier-Forsyth, 1994). The 5′-ΔC1-3/4 $tRNA^{Pro}$ transcript (57-mer) was prepared using a *Taq* I linearized plasmid encoding the $tRNA^{Pro}$ gene. Deletion of the nucleotide at the 5′ end of $tRNA^{Pro}$, C1, facilitates in vitro transcription and does not significantly affect aminoacylation by ProRS (Liu & Musier-Forsyth, 1994; Liu et al., 1995). Full-length and 3/4-tRNAs were purified on 12% polyacrylamide gels.

For the determination of RNA concentrations, the following extinction coefficients were employed: 76-mer, $60.4 \times 10^4\ M^{-1}$; 59-mer, $47 \times 10^4\ M^{-1}$; 57-mer, $41.8 \times 10^4\ M^{-1}$; 16-mer, $13 \times 10^4\ M^{-1}$; and 15-mer, $12 \times 10^4\ M^{-1}$. These values were determined experimentally as described previously (Musier-Forsyth et al., 1991).

Aminoacylation assays

Prior to aminoacylation assays, semi-synthetic tRNAs were annealed as follows. The chemically synthesized 3′-16-mer was mixed with a stoichiometric amount of the in vitro transcribed 5′-3/4 tRNA in 50 mM HEPES, pH 7.5, and heated at 60 °C for 3 min. $MgCl_2$ was then added to 10 mM and the mixture was cooled to room temperature and placed on ice. An identical procedure was followed for the 3′-3/4 tRNA, except that a 1.5-fold excess of the 5′-15-mer oligonucleotide was used (Liu & Musier-Forsyth, 1994). The annealed tRNAs (1–8 μM) were then assayed with purified ProRS essentially as described (Liu & Musier-Forsyth, 1994). Native gel electrophoresis of annealed tRNA molecules was also performed as described (Liu & Musier-Forsyth, 1994).

Chemical modification and primer extension

Modification with DMS and kethoxal was carried out at room temperature by reaction of 0.5% DMS (Aldrich) for 5 min or 0.04% kethoxal (ICN Biomedical) for 10 min with 80 pmol tRNA annealed in 10 mM $MgCl_2$, 50 mM sodium cacodylate, pH 7.0, and 100 mM KCl (Hou, 1994). The DMS reaction was quenched by placing on ice and adding β-mercaptoethanol to a final concentration of 200 mM (Pyle & Cech, 1991). The kethoxal reaction was stopped by adding KOH-borate, pH 7.0, to 15 mM (Hou, 1994), followed by ethanol precipitation. The modified tRNAs were denatured and hybridized with a 5′-^{32}P-end-labeled DNA primer complementary to nucleotides A59–A76 of $tRNA^{Pro}$. Primer extension was performed as described (Inoue & Cech, 1985), using AMV reverse transcriptase (USB Life Sciences) at 42 °C for 5 min.

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L P Yap and K Musier-Forsyth

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