Mapping Contacts between *Escherichia coli* Alanyl tRNA Synthetase and 2′ Hydroxyls Using a Complete tRNA Molecule†

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ABSTRACT: A dual-specific derivative of yeast $tRNA^{Phe}$ is described whose features facilitate structure−function studies of tRNAs. This tRNA has been made in three different bimolecular forms that allow modifications to be easily introduced into any position within the molecule. A set of deoxynucleotide substituted versions of this tRNA has been created and used to examine contacts between tRNA and *Escherichia coli* alanyl-tRNA synthetase, an enzyme previously shown to interact with 2′-hydroxyls in the acceptor stem of the tRNA. Because the present experiments used a full-length tRNA, several contacts were identified that had not been previously found using microhelix substrates. Contacts at similar sites in the T-loop are seen in the cocrystal structure of $tRNA^{Ser}$ and *Thermus thermophilus* seryl-tRNA synthetase.

The emerging X-ray crystal structures of the ribosome and its subunits (*1*−*3*) create the need for tRNA substrates that contain specific atomic or functional group modifications for experiments that probe the mechanism of translation. One goal of this work was to develop a new tRNA molecule with structural features that maximize the potential for such detailed structure−function studies. The well-characterized yeast $tRNA^{Phe}$ was chosen as the starting point not only because it is the best characterized substrate in assays that determine the kinetic rate constants in the steps of translation (*4*) but also because its structure is very well understood. X-ray crystal structures of yeast $tRNA^{Phe}$ alone (*5*, *6*) or in complex with either *Thermus aquaticus* elongation factor Tu (*7*) or *Thermus thermophilus* PheRS (*8*) are available. In addition, a variety of spectroscopic and biochemical studies have examined the structure and dynamics of $tRNA^{Phe}$ in solution (*9*−*14*). Finally, the unmodified $tRNA^{Phe}$ and a large collection of mutants have been made and assayed for their activity with its cognate synthetase, elongation factor Tu, and the ribosome (*15*−*18*).

A complication in using yeast $tRNA^{Phe}$ derivatives in translation assays is that its cognate synthetase is likely to form extensive contacts with a large part of the molecule, making uncertain whether a given modified tRNA can be aminoacylated to permit its use in subsequent translation assays. Indeed, numerous $tRNA^{Phe}$ mutations that either disrupt the tertiary structure or modify the interface with the synthetase are poorly aminoacylated (*16*). Thus, an important feature in the design of the new tRNA was the introduction of the identity elements for a second synthetase so that a modified tRNA could still be aminoacylated even if it were a poor substrate of PheRS. The identity elements of *Escherichia coli* AlaRS[1] were chosen because they are limited to a region of the acceptor stem of the tRNA that is not needed for PheRS activity (*19*−*21*). The nonoverlapping sequence and structural requirements of the two synthetases should permit any derivative of the "dual-specific" $tRNA^{Phe/Ala}$ to be aminoacylated with at least one amino acid. An additional advantage of such a tRNA is that it would permit the analysis of derivatives of the terminal ribose moiety since AlaRS initially aminoacylates the 3′-hydroxyl while PheRS initially aminoacylates the 2′-hydroxyl (*22*).

Although many modifications can be specifically introduced into a tRNA by biochemical methods (*23*−*25*), the most versatile approach to obtain the broadest possible diversity of modifications is the use of chemical synthesis. However, while the methods for chemical synthesis of RNA have improved dramatically over the past several years (*26*, *27*), it remains both difficult and expensive to prepare large numbers of modified tRNAs by complete chemical synthesis. Thus, a strategy was chosen to make tRNAs in two fragments, thereby allowing the synthetic effort to be focused on a limited region of the molecule. Three different pairs of fragment lengths were chosen such that each bimolecular tRNA molecule would correctly fold into the tRNA tertiary structure (*28*). While these bimolecular tRNAs can be easily ligated to give an intact tRNA, we have found that they often retain full activity despite the presence of a nick in the phosphodiester backbone.

An initial use of this system, which illustrates the versatility of the method, was the synthesis of 44 tRNA derivatives, each containing a single deoxynucleotide substitution at a unique position. These variants allow the role of each 2′-hydroxyl to be individually examined at any of several steps in the tRNA life cycle. Such a modification was desired because the 2′-hydroxyls in tRNA, as well as the phosphate oxygens, are likely to be critical components for interactions with enzymes that must associate with all

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[1] Abbreviations: AlaRS, alanyl-tRNA synthetase; PheRS, phenylalanyl-tRNA synthetase; SerRS, seryl-tRNA synthetase.

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tRNAs. While the roles of chemical groups in the phosphodiester backbone of various RNAs have been previously examined using mixed populations of RNAs (*29*−*31*), such methodologies suffer from the inability to examine 2′-hydroxyl function in the absence of an adjacent phosphorothioate modification. In addition to overcoming this difficulty, the use of singly deoxynucleotide substituted tRNAs permits a more quantitative analysis of their biochemical effects (*31, 32*).

While this set of tRNAs was designed with the translational apparatus in mind, experiments presented here examined the roles of these 2′-hydroxyls in aminoacylation by *E. coli* AlaRS. By determining the rates of aminoacylation of these singly deoxy-substituted tRNAs, several discreet contacts between the tRNA and AlaRS were identified. These data were compared with similar experiments using a much smaller substrate of AlaRS that had proposed a number of contacts between the AlaRS and 2′-hydroxyls near the 5′- and 3′-ends of $tRNA^{Ala}$ (*33*). The locations of these positions also suggest the portion of the tRNA that is involved in an interface with the synthetase.

MATERIALS AND METHODS

Plasmid Construction. A plasmid containing the YFA2 gene behind a T7 promoter was created by PCR using plasmid DNA encoding yeast $tRNA^{Phe}$ (*34*) and PCR primers containing both the T7 promoter sequence and the five required nucleotide changes. Ligation of the PCR product into the *Sma*I site of pUC19 created the appropriate *Bst*N1 restriction site for runoff transcription by T7 RNA polymerase. This same plasmid was initially used for production of the 57-nucleotide 5′-fragment of YFA2 by runoff transcription after digestion with *Taq*$^{\alpha}$1 endonuclease. However, this endonuclease required that the plasmids be grown in a *dam*$^{-}$ *E. coli* strain (such as GM2163), which resulted in poor plasmid yields. Additionally, in vitro transcription using plasmids purified from these cells yielded significantly less product than usual. These problems were solved by making a new plasmid, YFA2 (U59A), which created a unique *Bst*B1 restriction site, allowing for runoff transcription to give the same 57-nucleotide fragment with significantly higher yields. Large amounts of this plasmid can be obtained using an *E. coli* XL1-Blue host (Stratagene). PCR was used to create a plasmid containing the T7 promoter sequence directly linked to nucleotide G18 in the YFA2 sequence. When cleaved with *Bst*N1, transcription from this plasmid yields an RNA fragment containing the 3′-terminal 59 nucleotides of YFA2.

RNA Preparation. All transcription reactions were performed under the following conditions: 50 mM Tris-HCl (pH 8.1), 50 mM NaCl, 2.5 mM each NTP, 20 mM GMP, 30 mM $MgCl_2$, 1 mM spermidine, 0.1 mg/mL plasmid DNA, and 0.03 mg/mL T7 RNA polymerase. The inclusion of GMP in the reaction ensured that virtually all of the RNA products contained a monophosphate at their 5′-ends. Products of the transcription reactions were purified on 12% PAGE. Oligoribonucleotides 17 and 19 nucleotides in length were synthesized by standard procedures (*35*) using phosphoramidites purchased from Glen Research and terminated with 5′-hydroxyls. The 38-nucleotide RNA fragments representing the 5′-half (terminated with a 5′-hydroxyl) and 3′-half (terminated with a 5′-phosphate) of YFA2 were synthesized using ACE technology (*27*) (Dharmacon Research). All chemically synthesized oligonucleotides were purified on denaturing 15% polyacrylamide gels.

YFA2 (17), the bimolecular tRNA containing a nick after position 17, was formed by combining the 17-nucleotide 5′-fragment at a concentration of 30 μM with the 59 nucleotide 3′-fragment at 10 μM in 30 mM HEPES (pH 7.5) and 30 mM KCl. This mixture was heated to 65 °C for 3 min, followed by addition of $MgCl_2$ to a final concentration of 15 mM and subsequently cooled to room temperature over 30 min. Formation of YFA2 (57), the bimolecular tRNA containing a nick after position 57, was achieved by annealing the 57-nucleotide 5′-fragment at 1 μM with the 19-nucleotide 3′-fragment also at 1 μM using the same protocol as above. Formation of YFA2 (38), the bimolecular tRNA containing a nick after position 38, was accomplished by combining each RNA fragment at a concentration of 1 μM in 30 mM HEPES (pH 7.5) and 30 mM KCl. This mixture was heated to 90 °C for 3 min and then immediately placed on ice for 5 min. The sample was subsequently heated to 65 °C for 3 min followed by the addition of $MgCl_2$ to a final concentration of 15 mM, then slowly cooled to room temperature. Annealing of all three bimolecular tRNAs was verified using native gel electrophoresis, performed at room temperature on 10% polyacrylamide gels containing 30 mM Tris-acetate (pH 7.5), and 15 mM $MgCl_2$.

When desired, ligation of the bimolecular tRNAs to form full-length tRNAs was achieved by the addition of 1 mM ATP and 1 U/μL T4 RNA ligase to the preannealed RNAs, followed by a 3-h incubation at 17 °C. Ligation of YFA2 (17) and YFA2 (38) required no additional treatment prior to ligation, as both nicked sites contained a free 3′-hydroxyl and 5′-phosphate. However, because the 19 nucleotide 3′ fragment of YFA2 (57) was terminated with a free 5′-hydroxyl, this fragment was phosphorylated using T4 polynucleotide kinase prior to annealing to its 5′-counterpart.

Kinetic Characterization. Full-length, histidine-tagged *E. coli* AlaRS was purified from plasmid pQE-*alaS*-6H (*36*) as previously described (*37*). The individual kinetic constants for aminoacylation by AlaRS were determined by measuring activity at 37 °C in a reaction containing 30 mM HEPES (pH 7.5), 30 mM KCl, 15 mM $MgCl_2$, 4 mM ATP, 5 mM DTT, and 20 μM ^{3}H-alanine (50 Ci/mmol). Yeast PheRS was purified from baker's yeast as previously described (*38*). Individual kinetic constants for aminoacylation by PheRS were determined by measuring activity at 37 °C in a reaction containing 30 mM HEPES (pH 7.5), 30 mM KCl, 15 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, and 10 μM ^{3}H-phenylalanine (65 Ci/mmol). Depending on the substrate, RNA concentrations were varied between 0.02 and 5 μM, while AlaRS concentrations ranged from 2 to 50 nM, and PheRS concentrations ranged from 1 to 5 nM. Each deoxynucleotide-substituted tRNA was analyzed under a single set of conditions designed to measure k_{cat}/K_M.

Aminoacylation was measured by removing reaction aliquots at various time intervals and quenching in one of two ways. First, using the more traditional method, reaction aliquots were spotted directly onto Whatman 3MM paper presoaked in 10% trichloroacetic acid (TCA), which was then washed in 5% TCA, with the remaining radioactivity determined by liquid scintillation counting (*23*). In method two, reaction aliquots were quenched by the addition into

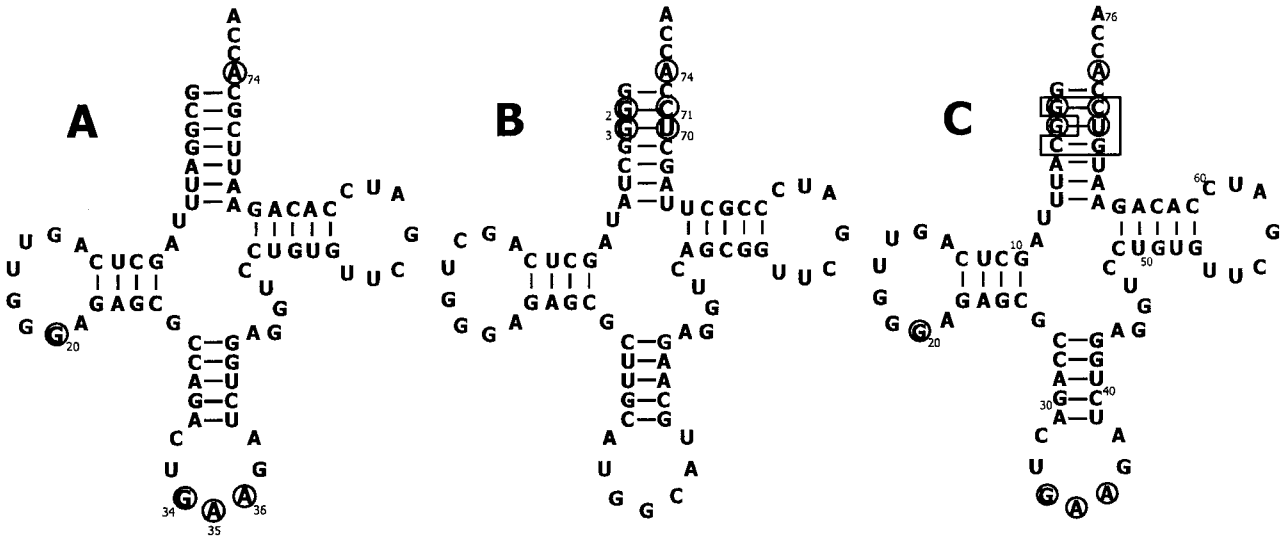

FIGURE 1: Secondary structures of (A) YF0, the transcript of yeast $tRNA^{Phe}$, (B) CA0, the transcript of *E. coli* $tRNA^{Ala}$, and (C) YFA2. Circled nucleotides indicate nucleotides required for aminoacylation by the cognate synthetase. Boxed nucleotides in YFA2 indicate the nucleotides changed from YF0.

100 μL of 5% TCA containing 10 μg of unfractionated tRNA as carrier. These samples were collected on Millipore HAWP filters using a modified dot-blot (Schleicher and Schuell) filtration apparatus (*39*) and washed with 6 × 100 μL aliquots of 5% TCA. After the entire filter was washed in 95% ethanol, the remaining radioactivity on the dried filter was then determined by exposure for ≥12 h to a tritium-capable phosphorimager screen (Molecular Dynamics). While identical results were obtained using each method, development of the second method was significant for two reasons. First, it allowed for the use of multichannel pipettors that dramatically increased sample throughput, and second, the use of the phosphorimager screen allowed for significantly higher sensitivity to the tritium signal than the liquid scintillation counting. Values of k_{cat}/K_M varied by <10% for duplicate samples and <20% when determined using different enzyme and/or tRNA concentrations.

RESULTS

A Dual-Specific tRNA. A derivative of yeast $tRNA^{Phe}$ was required that contained a limited number of changes such that it could be easily aminoacylated with *E. coli* AlaRS without losing its ability to be a substrate for yeast PheRS. As shown in Figure 1, the recognition nucleotides for in vitro aminoacylation by yeast PheRS (*40*) and *E. coli* AlaRS (*20*) lie in separate parts of the tRNA, suggesting that one tRNA might easily include both sets of determinants. This goal was accomplished by creating a new tRNA, termed YFA2 [for yeast phenylalanine (F) + alanine (A) tRNA − 2], that contains only five nucleotide changes from the sequence of yeast $tRNA^{Phe}$ (see Figure 1C). A derivative of $tRNA^{Phe}$ containing only three of these changes (C70U and C2G, G71C) had previously been shown to confer alanine identity on $tRNA^{Phe}$ (*15*). The additional G4C, C69G base pair change in YFA2 was included to reduce the 5′-end heterogeneity introduced by T7 RNA polymerase (*41*), resulting in a larger proportion of tRNA molecules with the correct sequence.

To confirm that these five changes conferred an efficient level of alanine identity onto this tRNA, its kinetic parameters for aminoacylation by *E. coli* AlaRS were measured and compared to those of CA0, the transcript of wild-type *E. coli* $tRNA^{Ala}$. As shown in Table 1, the values for k_{cat} and K_M are in close agreement, revealing only approximately a 2-fold decrease in k_{cat} and a 2-fold increase in K_M for YFA2. At least some of these small differences in aminoacylation between YFA2 and CA0 can likely be attributed to the introduction of the C4-G69 base pair. A similar base pair change introduced into the wild-type $tRNA^{Ala}$ sequence, making CA2 (*41*), resulted in a similar decrease in k_{cat} (see Table 1). It is important to note, however, that the slight decrease in kinetic efficiency was accompanied by a significant reduction in the production of deleterious 5′-end heterogeneity for these transcripts. The result of this reduction is a more homogeneous population of tRNA, devoid of the contaminating products that can alter the measured kinetic values (*41*).

Table 1: Kinetic Constants for Aminoacylation by *E. coli* AlaRS or Yeast PheRS

AlaRS	k_{cat} (s^{-1})	K_M ($\times 10^{-6}$ M)	k_{cat}/K_M ($\times 10^5$ M^{-1} s^{-1})
CA0	0.44	1.1	4.0
YFA2	0.25	2.1	1.2
CA2	0.19	1.1	1.7
PheRS	k_{cat} (s^{-1})	K_M ($\times 10^{-6}$ M)	k_{cat}/K_M ($\times 10^7$ M^{-1} s^{-1})
YF0	23	0.7	3.3
YFA2	22	0.7	3.1

While these changes were effective in making YFA2 a substrate for AlaRS, a similar comparison with yeast PheRS revealed virtually no loss in activity. Under conditions of optimal PheRS activity, the values for k_{cat} and K_M for phenylalanylation of YFA2 were found to be nearly identical to the values determined for YF0, the transcript of wild-type yeast $tRNA^{Phe}$ (see Table 1). These findings are consistent with previous experiments showing that mutations in the acceptor stem of yeast $tRNA^{Phe}$ had no effect on the parameters of aminoacylation with PheRS (*40*). The rate of lead-specific cleavage of YFA2 was also found to be identical to that of YF0 (data not shown). Since lead cleavage is a sensitive gauge of the tertiary folding of a tRNA (*42*), this

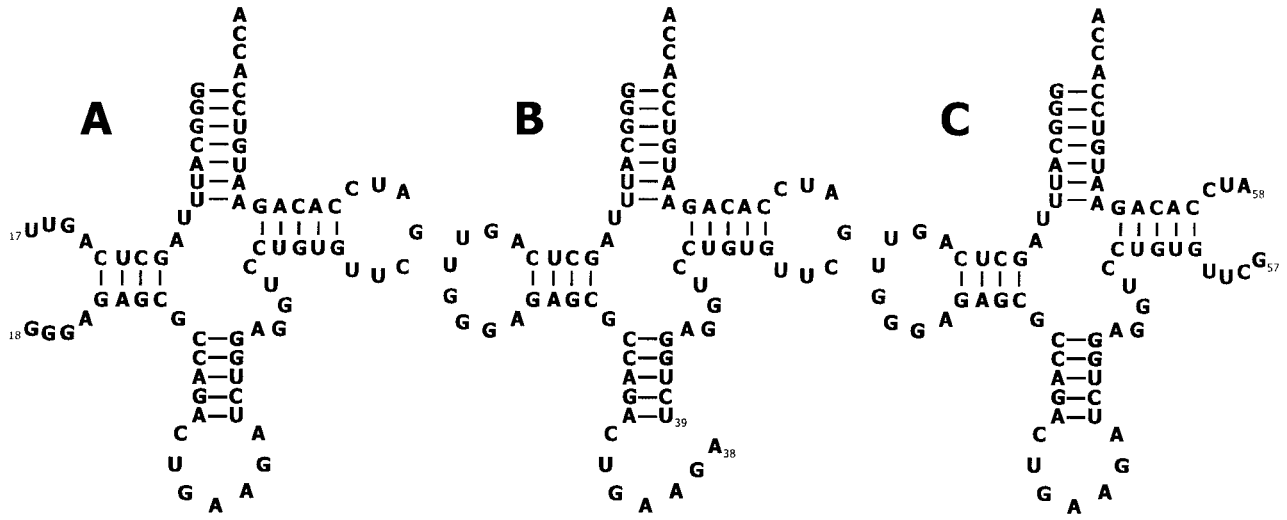

FIGURE 2: Bimolecular tRNAs (A) YFA2 (17), (B) YFA2 (38), and (C) YFA2 (57).

experiment suggests that the changes in the acceptor stem did not alter the overall tertiary structure of the molecule.

Bimolecular Versions of YFA2. To simplify the introduction of site-specific modifications of YFA2 by chemical synthesis, the tRNA molecule was prepared by annealing two oligonucleotides to create bimolecular tRNAs that contained a nick in the tRNA backbone. Three different versions of YFA2 were created in which the backbone was interrupted on the 3′-side of nucleotides 17, 38, and 57, creating YFA2 (17), YFA2 (38), and YFA2 (57), respectively (see Figure 2). The sites for the nicks were chosen to be in the tRNA loops to maximize the likelihood that the two fragments could be joined by T4 RNA ligase (*23*). In addition, all three nicks were at backbone positions where a nick could be introduced without disrupting the tertiary folding of $tRNA^{Phe}$ (*28*).

The bimolecular YFA2 (17) and YFA2 (57) molecules were designed such that the small fragment could be made by chemical synthesis and the large fragment by transcription with T7 RNA polymerase. An important consideration for the choice of a nick after position 17 was the presence of a good transcription initiation site for T7 polymerase starting with nucleotide 18, permitting efficient transcription of the large fragment. Similarly, a nick at position 57 was initially chosen because a *Taq*$^{\alpha}$1 endonuclease restriction site appears at that position in the YFA2 gene, allowing for convenient runoff transcription of that fragment. A new plasmid was subsequently constructed that allows for production of the same 57-nucleotide fragment using a *Bst*B1 restriction site, greatly improving both plasmid and transcription yields (see Materials and Methods). Finally, the bimolecular YFA2 (38) tRNA takes advantage of improvements in the chemical synthesis of RNA that permit efficient synthesis of the two 38-nucleotide fragments (*27*). While this bimolecular tRNA permits the introduction of modifications into any part of the tRNA by chemical synthesis, significantly more synthetic effort is required for this bimolecular tRNA than for the other two versions.

The annealing of the two oligonucleotides to form each bimolecular tRNA was monitored by native gel electrophoresis. Although slightly different salt and metal ion concentrations were required to optimize annealing, efficient formation of both YFA2 (38) and YFA2 (57) was accomplished by combining equimolar amounts of the two oligonucleotides. Because of the presence of competing structures, the optimized protocol for formation of YFA2 (17) required a 3-fold excess of the smaller 5′-fragment to fully anneal with the large 3′-fragment. While efficient formation of the complexes required relatively high RNA concentrations (1−10 μM), all of the bimolecular tRNAs studied here could subsequently be diluted to sub-nanomolar concentrations and remained intact during incubation at 37 °C.

Table 2: Kinetic Constants for Aminoacylation of Bimolecular tRNAs

AlaRS	k_{cat} (s^{-1})	K_M ($\times 10^{-6}$ M)	k_{cat}/K_M ($\times 10^5$ M^{-1} s^{-1})
YFA2	0.25	2.1	1.2
YFA2 (17)	0.25	1.9	1.3
YFA2 (38)	0.46	3.1	1.5
YFA2 (57)	0.12	17	0.07
PheRS	k_{cat} (sec^{-1})	K_M ($\times 10^{-6}$ M)	k_{cat}/K_M ($\times 10^6$ M^{-1} s^{-1})
YFA2	22	0.7	31
YFA2 (17)	9.5	0.5	19
YFA2 (38)	0.9	4.0	0.23
YFA2 (57)	19	0.9	21

Ligation of the three nicked tRNAs to form intact tRNAs was monitored on denaturing 10% polyacrylamide gels. Using low concentrations of T4 RNA ligase with an existing protocol (*23*), both YFA2 (38) and YFA2 (57) were efficiently converted to a single species that matched the mobility of the full-length YFA2 (data not shown). Under these same conditions, YFA2 (17) was a poor substrate for RNA ligase, presumably due to the location of the nick in the folded structure of the tRNA. Improved ligation yields for this tRNA can be achieved through the use of a DNA splint and T4 DNA ligase (*43*).

While the bimolecular versions of YFA2 were designed to simplify the ligation process, an examination of the activity of the nicked versions suggested that the ligation step was not always necessary. As shown in Table 2, the kinetic constants for aminoacylation of both YFA2 (17) and YFA2 (38) with AlaRS are virtually unchanged from those of intact tRNA. However, while YFA2 (57) showed no significant change in the value for k_{cat}, it did show an increase in K_M. A similar change in K_M was seen when a nick was introduced

in the backbone of CA0 after position 57 (data not shown). The corresponding kinetic constants for aminoacylation by PheRS suggest that the nicks after positions 17 and 57 have virtually no effect, while the nick after position 38 showed a significant reduction in k_{cat} and an increase in K_M. Contrary to a previous report (*44*), no activity was observed for the 3′-terminal 59-nucleotide fragment by itself with PheRS.

2′-Hydroxyl Modifications with AlaRS. One type of modification expected to affect the interaction of tRNA with many enzymes is the substitution of 2′-hydroxyls with protons. As a means of examining such effects, oligonucleotides were chemically synthesized that individually replaced each ribose sugar with a deoxyribose sugar in the smaller portion of YFA2 (57) and at all but the 3′-terminal position in YFA2 (17). Additionally, deoxynucleotides were introduced at positions 49−57 using the YFA2 (38) system. The resulting 44 different YFA2 molecules permit every 2′-hydroxyl in the acceptor arm domain of the tRNA to be individually examined for its role in tRNA function.

Each of these modified bimolecular tRNAs was assayed for its activity with *E. coli* AlaRS, an enzyme previously shown to interact predominantly with this upper portion of the tRNA (*21*). Experiments were designed to compare the initial rate of aminoacylation for each of the deoxy-substituted variants with that of an all-RNA version of the appropriate bimolecular control. Measurements were made under conditions in which the tRNA concentration was approximately 10-fold lower than the K_M, requiring slightly different conditions for each of the bimolecular tRNAs. At least three independent measurements were made for each variant, with the results of the experiments shown in Table 3. Significantly, 31 of the 44 modified tRNAs had nearly identical kinetic parameters as the all-RNA controls, suggesting that single deoxynucleotide substitutions do not generally affect RNA folding. However, substitution at positions 70, 71, 75, or 76 in YFA2 (57) resulted in a decrease of over 100-fold in the rate of aminoacylation. Additionally, substitution at positions 2, 4, 56, 57, 69, or 74 resulted in more modest 2−10-fold decreases in aminoacylation, while substitutions at positions 49 or 72 resulted in a small increase in the rate of aminoacylation. It is important to note that all of the variants that displayed an altered activity in these studies contained a deoxynucleotide substitution that was located well away from the nick in the tRNA. For example, the d56 and d57 variants were identified using bimolecular tRNAs containing a nick in the anticodon and not those containing a nick at position 57. The observed $\Delta\Delta G^{\ddagger}$ at these positions are therefore be likely to be independent of the presence of the nick sites. The absence of an observed effect for deoxynucleotide substitutions located near a nick site, such as d58 or d16, should be considered less reliable by virtue of their proximity to the nick sites.

To test whether the altered activity displayed by these 13 variants with AlaRS was a result of changes in the folded structure of YFA2, they were assayed with PheRS, an enzyme that is very sensitive to changes in the folded state of tRNA. Eleven of the 13 variants showed wild-type activity with PheRS, one (d76) could not be examined with PheRS because the 2′-hydroxyl is required as the nucleophile in the aminoacylation reaction, and one (d74) showed a modest decrease in activity with both enzymes (data not shown).

Table 3: Effects of Deoxynucleotide Substitutions in Bimolecular YFA2 Substrates on the Activity of *E. coli* AlaRS[a]

	k_{cat}/K_M ($\times 10^5$ M^{-1} s^{-1})	$\Delta\Delta G^{\ddagger}$ (kcal/mol)[b]
YFA2 (17)	1.3	
d1	1.2	0.0
d2	0.6	0.5
d3	1.4	0.0
d4	0.7	0.4
d5	1.7	−0.2
d6	1.3	0.0
d7	1.1	0.1
d8	0.9	0.2
d9	1.0	0.2
d10	1.2	0.0
d11	1.3	0.0
d12	1.4	0.0
d13	1.4	−0.1
d14	1.6	−0.1
d15	1.4	0.0
d16	1.5	−0.1
YFA2 (38)	1.2	
d49	2.3	−0.4
d50	1.1	0.0
d51	1.1	0.1
d52	1.4	−0.1
d53	1.0	0.1
d54	1.0	0.1
d55	1.0	0.1
d56	0.43	0.6
d57	0.56	0.5
	k_{cat}/K_M ($\times 10^3$ M^{-1} s^{-1})	$\Delta\Delta G^{\ddagger}$ (kcal/mol)[b]
YFA2 (57)	7.0	
d58	6.3	0.1
d59	7.2	0.0
d60	6.8	0.0
d61	6.0	0.1
d62	6.9	0.0
d63	6.6	0.0
d64	6.0	0.1
d65	6.9	0.0
d66	8.1	−0.1
d67	6.6	0.0
d68	7.7	−0.1
d69	5.3	0.2
d70	≤0.07	≥2.8
d71	≤0.07	≥2.8
d72	14	−0.4
d73	6.5	0.1
d74	2.0	0.8
d75	≤0.07	≥2.8
d76	≤0.07	≥2.8

[a] Aminoacylation was measured as detailed in Materials and Methods. [b] $\Delta\Delta G^{\ddagger} = -RT \ln[(k_{cat}/K_M)^{variant}/(k_{cat}/K_M)^{control}]$.

Taken together, these data suggest that the change in activity of the single deoxynucleotide substitutions is unlikely to be the result of a change in the folded structure of YFA2 but is rather the result of disrupting a contact with the synthetase. Presumably, the d74 modification alters an interaction made with both enzymes.

DISCUSSION

A critical decision in planning detailed structure−function studies is the choice of a "wild-type" molecule to be used as the basis for comparison of the many modified molecules to be tested. While yeast $tRNA^{Phe}$ is an obvious choice for a tRNA to use in experiments on the ribosome, the variant

described here contains five nucleotide changes in the acceptor stem that also make the molecule a substrate for *E. coli* AlaRS. While a number of other dual-specific tRNAs have been designed as a means of examining tRNA identity (*15*, *45*, *46*), the inclusion of the alanine identity elements in this tRNA was simply intended to facilitate the production of aminoacyl tRNAs for use in structure−function studies. Since AlaRS can aminoacylate almost any RNA substrate with an appropriate acceptor stem, it should be possible to alanylate virtually any desired modifications of $tRNA^{Phe}$. For the limited number of molecules that are not substrates for AlaRS, the orthogonal substrate requirements of PheRS make it likely that they can be phenylalanylated. Furthermore, many modifications of this dual-specific tRNA should be efficiently aminoacylated by both PheRS and AlaRS, allowing the identity of the amino acid to be varied, a feature that may be useful for some applications. An additional benefit of this dual-specific tRNA is its ability to be specifically aminoacylated on either the 2′- or 3′-terminal hydroxyl. This functionality will be useful in future experiments aimed at examining the chemical reactivity of the aminoacyl linkage during various steps of translation.

The decision to prepare modified tRNAs using a combined chemical and enzymatic synthesis strategy reflects the current status of methodologies for RNA synthesis. Solid-phase methods of chemical synthesis offer the best opportunity to produce a diversity of modified RNAs, yet suffer from their inability to efficiently make long molecules as well as their requirement for expensive reagents. Enzymatic methods can produce longer RNAs efficiently, but protocols for introducing selective modifications are complicated. By focusing the synthetic efforts on a short region of the molecule, a large, diverse collection of modified tRNAs can be made while still realizing the benefits of the enzymatic methods.

While the initial intent was to use RNA or DNA ligase to join the two oligonucleotides and form an intact tRNA, it became clear that this would not always be necessary. Since the nick sites were chosen so as not to affect the folding of the tRNA, and the two oligonucleotides remain annealed under most assay conditions, it is possible to perform many experiments using bimolecular tRNAs. Indeed, all three of the bimolecular forms of YFA2 are active substrates with AlaRS, and two of these are fully active substrates with PheRS. Presumably when the nick is well away from the region of the tRNA that interacts with the protein, the enzyme reaction is not affected. The decreased activity of YFA2 (38) with PheRS can be explained by the fact that this enzyme makes multiple contacts with anticodon residues (*40*, *47*). Since the anticodon residues are not likely to be perfectly ordered in YFA2 (38) before it binds the enzyme, some additional binding free energy is presumably needed to organize these residues in the bound state. In addition, it is possible that the replacement in the nicked tRNAs of a phosphodiester linkage, containing only a single negative charge, with a monophosphate containing two negative charges and a free 3′-hydroxyl could affect the integrity of the interaction.

The observation that nicked tRNAs can be active in aminoacylation reactions has been known for 30 years (*48*−*50*). In a particularly careful recent analysis, Musier-Forsyth and colleagues showed that *E. coli* $tRNA^{Pro}$ with nicks after either nucleotide 17 or 57 retained almost full activity with ProRS (*32*, *51*). We have also found that transcripts of *E. coli* $tRNA^{Ala}$ with nicks after positions 17 or 57 display nearly wild-type activity with *E. coli* AlaRS (data not shown). It also seems likely that nicked tRNAs will be efficient substrates for many other enzymes. We have in fact found that all three nicked forms of YFA2 interact normally with both *T. thermophilus* elongation factor Tu and wheat germ elongation factor 1-α (J.A.P. and O.C.U., unpublished results). The advantage of using nicked tRNAs in structure−function studies is the reduced time required to make modified tRNA molecules. There are, however, certain unique applications that depend on the activity of the nicked tRNA. For example, YFA2 (57) has been used to develop an aminoacylation assay that uses an internal ^{32}P label to facilitate product and substrate separation and can be adapted for pre-steady-state measurements (*37*).

As an initial demonstration of the utility of the YFA2 system, a series of modified bimolecular tRNAs were prepared that contained single deoxynucleotide substitutions at each of 44 positions that cover the entire acceptor arm and T-stem domains of the tRNA. The deoxynucleotide modification was chosen for several reasons. First, the 2′-hydroxyl groups on the tRNA are on the outside of the molecule and hence are expected to form contacts with many enzymes that interact with tRNA, especially those that must interact with all tRNAs. For example, 2′-deoxy modified tRNAs should be valuable at probing the changing environment of the tRNA as it proceeds through the ribosome. Second, the 2′-deoxy substitution is structurally conservative in the sense that it will disrupt the ability of the 2′-hydroxyl to act as a hydrogen-bond donor or acceptor, but it will do so without introducing steric clashes that are possible with substitutions such as 2′-*O*-methyls. Finally, the thermodynamic consequences of single 2′-deoxynucleotide substitutions have already been examined in a variety of RNA−protein interactions (*31*, *52*−*55*) and give values of $\Delta\Delta G^{\ddagger}$ that are easily detected but not so large as to prevent measurement.

This set of deoxynucleotide substituted tRNAs was used to examine backbone interactions with *E. coli* AlaRS, an enzyme predicted to interact with the acceptor arm domain of a tRNA. The effect of these substitutions was assessed by comparing the k_{cat}/K_M for each of the individual variants with an all-RNA control (see Table 3). While limitations inherent to this and most other aminoacylation assays prevent the identification of the precise step in the reaction pathway at which these variants are deficient, most of the observed values of $\Delta\Delta G^{\ddagger}$ ranged from 0.2 to 1.5 kcal/mol, values consistent with disruption of a single hydrogen bond between the synthetase and the tRNA during the rate-limiting step. Effects with similar magnitudes were observed for certain deoxynucleotide substitutions in both $tRNA^{Asp}$ (*31*) and $tRNA^{Pro}$ (*32*). The fact that most of these variants displayed full activity with PheRS suggests that the decreased activity observed with AlaRS is not the result of global misfolding of the tRNA but rather reflects the loss of a contact with the enzyme. Nevertheless, in the absence of high-resolution structural data for this complex, the exact nature of the disrupted interaction cannot be determined. While the simplest explanation is disruption of a direct hydrogen bond between the protein and the RNA, other explanations cannot be excluded. For example, these modifications may interfere

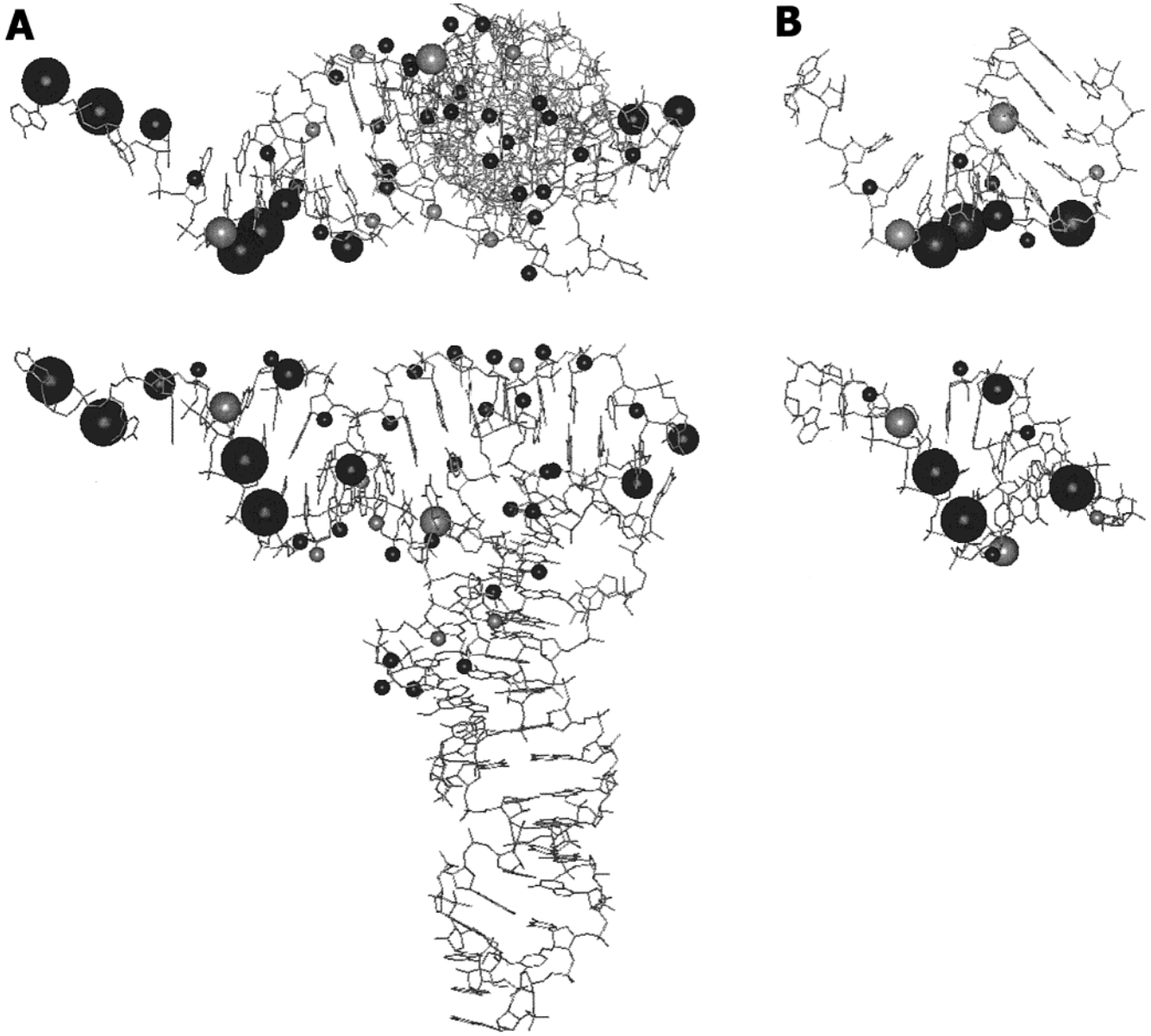

FIGURE 3: Two different three-dimensional depictions, related to one another by a 90° rotation about the *x* axis, of 2′-hydroxyl substitution effects in YFA2 determined here (A) and in a duplex RNA representing the first seven base pairs of $tRNA^{Ala}$ (*33*) (B). Diagrams were created using the coordinates from (A) the yeast $tRNA^{Phe}$ crystal structure (*5*) or (B) the NMR structure determined for a similar microhelix substrate (*74*). On these diagrams, the locations of each of the different 2′-hydroxyls examined in these studies are shown as balls. The size of the ball is proportional to the effect observed upon substitution with a deoxynucleotide, with the small balls representing $\Delta\Delta G^{\ddagger} \leq 0.2$ kcal/mol, the medium balls representing $\Delta\Delta G^{\ddagger}$ greater than 0.2 but less than 1.5 kcal/mol, and the large balls representing $\Delta\Delta G^{\ddagger} \geq 1.5$ kcal/mol. The direction of the change is denoted by the color of the ball, with negative values of $\Delta\Delta G^{\ddagger}$ shown in black and positive values in gray.

with metal-binding sites on the RNA that are critical for interactions with the synthetase. Likewise, the substitutions may change the solvent structure around the molecule, ultimately disrupting the interaction with the enzyme. A definitive explanation of these biochemical effects awaits the collection of additional structural information.

The larger energetic changes observed for the d70, d71, and d75 substitutions are not as easily understood as single hydrogen bonds. Instead, these large effects may indicate the involvement of these hydroxyls in a larger network of hydrogen bonds, possibly including a role in stabilizing the local solvation pattern. Such a role seems particularly intriguing around the area of the 3−70 base pair, where a tightly bound water molecule has been proposed (*56*, *57*). Alternatively, the proximity of these positions to the active site of the enzyme raises the possibility that they are involved not only in stabilizing the RNA−protein complex, but also in organizing the active site of the enzyme and thereby affecting the catalytic rate (*58*). While the large effect observed upon substitution of the 2′-hydroxyl at position 76 might also reflect disruption of a crucial interaction with the synthetase, some or perhaps all of the reduced rate is the result of the higher pK_a of the 2′-deoxyribose (*59*−*61*). Identification of the particular reaction step or steps at which this and other variants are deficient will require the development of a separate assay capable of measuring the individual rate constants under pre-steady-state conditions.

Those variants containing deoxynucleotide substitutions near the top of the acceptor stem can be compared with previous experiments that used a 9-base pair helix designed

to recreate the acceptor stem of $tRNA^{Ala}$ (*33*). In general, as shown in Figure 3, the results of the two studies are in good agreement. Seven of the 11 positions that can be directly compared show nearly identical values of $\Delta\Delta G^{\ddagger}$ with AlaRS. However, at positions 70 and 71 the magnitudes of the effects are significantly different. The present study determined $\Delta\Delta G^{\ddagger}$ values of $\geq$2.8 kcal/mol for each of these positions, while the previous study measured values of 1.7 kcal/mol for these same positions. Additionally, the previous study identified two positions, d4 and d68, that displayed significant changes from wild type ($\Delta\Delta G^{\ddagger}$ values of 1.2 and −0.4 kcal/mol, respectively). However, the current study found very little change at these positions ($\Delta\Delta G^{\ddagger}$ values of 0.4 and −0.1 kcal/mol, respectively). The reason for these differences remains unclear. While somewhat unlikely, these differences may simply reflect the small differences in the reaction conditions, such as temperature or amino acid concentration. Alternatively, the observed differences may be the result of the different RNA sequences used in these studies. It has been suggested that recognition by AlaRS of its substrate tRNA occurs by indirect recognition of the helical irregularities resulting from the G−U base pair (*62*). In this regard, it is intriguing to note that two of the positions that display significant discrepancies are 4 and 68, nucleotides that differ in identity between the two substrates.

Deoxynucleotide substitutions at positions 56 and 57 in the bimolecular YFA2 (38) also displayed decreased activity with *E. coli* AlaRS, suggesting an interaction with the synthetase in the T-loop region of the tRNA. These contacts are likely to explain the decreased efficiency of the bimolecular YFA2 (57) substrate when compared to YFA2. The nick in the phosphodiester backbone at this site would be expected to leave the T-loop somewhat disordered in the unbound form, causing a loss in binding energy. The fact that AlaRS interacts with the T-loop of the tRNA also explains the decreased activity as compared to the intact tRNA of the shorter microhelix and tetraloop substrates that completely lack a T-stem or loop (*21*, *63*). The inability of the shorter substrates to satisfy all of the interactions with the enzyme may also partially explain the discrepancies between the different deoxynucleotide substitution studies presented above. Because the smaller substrates used in the previous experiments are unable to fill the entire binding site on the synthetase, the interactions observed with these substrates may not precisely resemble their full-length counterparts.

Generally, it has been believed that the T-loop is not essential for AlaRS activity. This belief was in part the result of experiments that showed little or no loss of the activity when the T-stem and loop of the minihelix substrates was removed to form the microhelix substrates (*64*). However, even though the minihelix contains a T-stem and loop, the relative geometry of this part of the molecule is different from a full-length tRNA. In the minihelix, the acceptor and T-stems are coaxial with one another, whereas in the full-length tRNA they are bent by about 14° relative to one another (*65*). This may result in an inability of the minihelix substrate to properly position itself on the enzyme such that it can simultaneously satisfy interactions with both the T-loop and acceptor stem of the molecule.

While the three-dimensional structure of the complex between AlaRS and its substrate tRNA is not known, the results of these deoxynucleotide substitution experiments, when superimposed onto the three-dimensional structure of $tRNA^{Phe}$ (Figure 3A), can be used as a thermodynamic means to display where AlaRS interacts with the RNA. As a class II synthetase, it has been proposed that recognition by AlaRS of its substrate tRNA would be achieved in a similar fashion as other class II aminoacyl-tRNA synthetases (*66*). While cocrystal structures of four class II synthetases are available (*8*, *67*−*69*), the most compelling comparison comes from that of SerRS. Like AlaRS, SerRS has no required elements for aminoacylation in the anticodon of the tRNA. The cocrystal structure of SerRS indicates that most of the contacts made between the protein and the RNA are with elements of the phosphodiester backbone. Significantly, the structure reveals a long coiled-coil domain of the synthetase stretching down the acceptor arm of the tRNA, making contacts with residues in the variable loop as well as the T-loop of the tRNA, including the phosphodiester backbone of nucleotide 57. The observed decreases in aminoacylation by AlaRS upon deoxy-substitution of nucleotides 56 and 57 in YFA2 suggests that AlaRS may have a similar extension or domain interacting with the T-loop. However, the structure of AlaRS must certainly deviate from that of SerRS and the other class II structures, as the 3−70 base pair, which is critical for AlaRS activity, is entirely exposed to solvent in each of these. It has been suggested that a 76-amino acid appendage present in AlaRS is responsible for the recognition of the minor groove around this base pair (*70*).

We expect that the YFA2 tRNA in general, and this set of deoxy-substituted molecules in particular, will be useful reagents for evaluating numerous aspects of tRNA activity. In addition to facilitating the production of aminoacyl tRNAs, the dual-specific nature of YFA2 should allow for a dissection of the relative contributions of the aminoacyl versus RNA moieties in ribosome activity. Further, the ability to easily and specifically modify any position in the tRNA using these bimolecular tRNAs greatly expands the number of experiments available to the researcher. Finally, the set of deoxy-substituted YFA2 molecules described here should be immediately useful for examining interactions with enzymes such as elongation factor Tu or the large subunit of the ribosome, each of which has been shown to interact almost exclusively with the acceptor arm region of the tRNA (*71*−*73*).

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