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Specific Interaction of Anticodon Loop Residues with Yeast Phenylalanyl-tRNA Synthetase†

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ABSTRACT: Thirteen different yeast $tRNA^{Phe}$ variants with single nucleotide changes in positions 34–37 in the anticodon region were prepared by an enzymatic procedure described previously. Aminoacylation kinetics using purified yeast phenylalanyl-tRNA synthetase revealed that the level of aminoacylation was very different for different sequences inserted. The low level of aminoacylation was the result of a steady state between a slow forward reaction rate and spontaneous deacylation of the product. Aminoacylation kinetics performed at higher synthetase concentrations revealed that substitution at position 34 in $tRNA^{Phe}$ decreased the K_m nearly 10-fold but only had a small effect on V_{max}. Similar substitutions at positions 35, 36, and 37 had a lesser effect. These data suggest a sequence-specific contact between the anticodon of yeast $tRNA^{Phe}$ and the cognate synthetase.

In an early investigation on the interaction of yeast $tRNA^{Phe}$ with its cognate aminoacyl-tRNA synthetase, Thiebe et al. (1972) showed that the removal of four nucleotides (residues 34–37) from the anticodon loop of $tRNA^{Phe}$ did not completely destroy its ability to be aminoacylated. This experiment suggested that nucleotides in the anticodon loop were not involved in the specific protein–nucleic acid interaction. The view was supported by the data of Roe et al. (1973), who found that heterologous tRNAs with a variety of anticodon loop sequences could be aminoacylated by PRS.[1] Several other experiments, however, seemed to reach the opposite conclusion. By use of methods of fluorescence quenching (Krauss et al., 1973), oligonucleotide binding (Barrett et al., 1974), chemical modification with kethoxal (Litt & Greenspan, 1972), nuclease protection (Hörz & Zachau, 1973), and photochemical cross-linking (Ebel et al., 1979), the anticodon region was shown to interact with PRS.

Recently we have developed an enzymatic procedure which allows the removal of residues 34–37 from $tRNA^{Phe}$ and replacement of them with any arbitrary oligoribonucleotide (Bruce & Uhlenbeck, 1982). In preliminary experiments we noted that replacement of the $G_mpApApY$ sequence in $tRNA^{Phe}$ with ApApApC results in a tRNA which is much less active in the aminoacylation reaction. In this work we have prepared a series of tRNAs which systematically replace each of the four nucleotides and examine which positions are primarily responsible for the poor aminoacylation and therefore are presumably involved in the interaction with PRS.

Materials and Methods

Materials. Dinucleoside monophosphates and nucleoside 5′-diphosphates were purchased from Sigma Chemical Co. [5′-^{32}P]pCp was prepared from [γ-^{32}P]ATP (England et al., 1980) which was prepared from radioactive phosphate (Johnson & Walseth, 1978). L-[^{3}H]Phenylalanine (36.7 Ci/mmol) was purchased from Amersham. Yeast $tRNA^{Phe}$ was purchased from Boehringer-Mannheim (lot 1199234) and used directly for anticodon loop substitution or treated with tRNA nucleotidyltransferase and purified by gel electrophoresis for use in aminoacylation reactions.

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[1] Abbreviations: $tRNA^{Phe}$, yeast phenylalanine-accepting tRNA; $tRNA^{Phe}_{AAAG}$, a $tRNA^{Phe}$ molecule which has ApApApG substituted for the anticodon loop nucleotides $G_mpApApY$ (positions 34–37); PRS, yeast phenylalanyl-tRNA synthetase; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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T4 RNA ligase and T4 polynucleotide kinase were purified by the methods of Moseman-McCoy et al. (1979) and Cameron & Uhlenbeck (1977). Highly purified rabbit liver tRNA nucleotidyltransferase (Deutscher & Masiakowski, 1978) and elongation factor Tu (Miller & Weissbach, 1970) were gifts of M. Deutscher and T. Yamane. Homogeneous yeast phenylalanyl-tRNA synthetase with a specific activity of 3200 units/mg (Schmidt et al., 1971) was a gift of B. Reid. Primer-dependent *Micrococcus luteus* polynucleotide phosphorylase was purified by a modification of the procedure of Klee (1971). Bacterial alkaline phosphatase and yeast inorganic pyrophosphatase were purchased from Worthington.

Oligoribonucleotide Synthesis. The tetranucleotide GpApApA and the trinucleotides GpApA, CpApA, UpApA, GpCpA, GpUpA, and GpApG were synthesized by using polynucleotide phosphorylase in the equilibrium reaction (Thach & Doty, 1965). A typical reaction (200 μL) containing 7 mM dinucleoside monophosphate, 11 mM nucleoside 5′-diphosphate, 10 mM $MgCl_2$, 0.4 M NaCl, 0.2 M Tris-HCl, pH 8.2, and 100 units/mL polynucleotide phosphorylase was incubated for 48 h at 37 °C. The trinucleotides GpApU and GpApC were prepared in a similar manner, but the reactions contained 40 mM nucleoside diphosphate and included 60 μg/mL ribonuclease A. After incubation each reaction mixture was heated to 100 °C for 2 min to inactivate the polynucleotide phosphorylase, and then bacterial alkaline phosphatase was added to 0.1 mg/mL and incubated 3 h at 37 °C to degrade unreacted nucleoside diphosphates and remove 3′-terminal phosphates. The products of oligomer synthesis reactions were purified by descending chromatography on Whatman 3MM paper using a 1:1 mixture of 1.0 M ammonium acetate, pH 7.0, and 95% ethanol. The oligonucleotide products were located by viewing under an ultraviolet lamp, cut from the paper chromatogram, washed with absolute ethanol to remove the ammonium acetate, and eluted with distilled water. The molar yield of dimer incorporated into product varied from about 15% for the equilibrium reactions to nearly 90% for the ribonuclease-assisted reactions.

The tetranucleotides GpApApC, GpApApU, ApApApG, CpApApG, and UpApApG were prepared from the trinucleotides synthesized above by the nuclease-assisted polynucleotide phosphorylase reaction. In this case, the reactions contained 1 mM trimer, 35 mM nucleoside diphosphate, 100 units/mL polynucleotide phosphorylase, and either 50 μg/mL RNase A or 25 μg/mL RNase T_1 in the same buffer as above. Incubation was for 8 h at 37 °C. The oligomers GpApApApG, GpApApG, GpApCpG, GpApUpG, GpCpApG, and GpUpApG were synthesized by using T4 RNA ligase to add guanosine 3′,5′-bisphosphate to the trimer or tetramer made previously. Reactions contained 0.5 mM trimer or tetramer, 1–2 mM guanosine 3′,5′-bisphosphate, 3 mM ATP, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 50 mM Hepes, pH 8.3, and 800 units/mL RNA ligase and were incubated for 3 h at 37 °C. Both the polynucleotide phosphorylase and the RNA ligase reactions were inactivated, treated with alkaline phosphatase, and purified by paper chromatography as above. The reaction yields varied from 60 to 90% of the trinucleotide incorporated into product.

The length of each oligonucleotide could be determined by its comigration with known markers of similar nucleotide composition on the paper chromatograms during purification. In addition, each oligonucleotide was tested for the presence of the correct 3′- and 5′-terminal nucleotide. This was done by labeling each oligomer at the 5′ terminus with [γ-^{32}P]ATP and polynucleotide kinase or at the 3′ terminus with [5′-^{32}P]pCp and RNA ligase. The labeled oligonucleotides were then digested with a mixture of ribonucleases T_1, T_2, and A and analyzed by two-dimensional chromatography on a cellulose thin-layer plate (Nishimura, 1979). Autoradiography of the plate identified the 5′-terminal nucleotide of the oligonucleotide as a [5′-^{32}P]nucleoside 3′,5′-bisphosphate and the 3′-terminal nucleotide as a [^{32}P]nucleoside 3′-monophosphate.

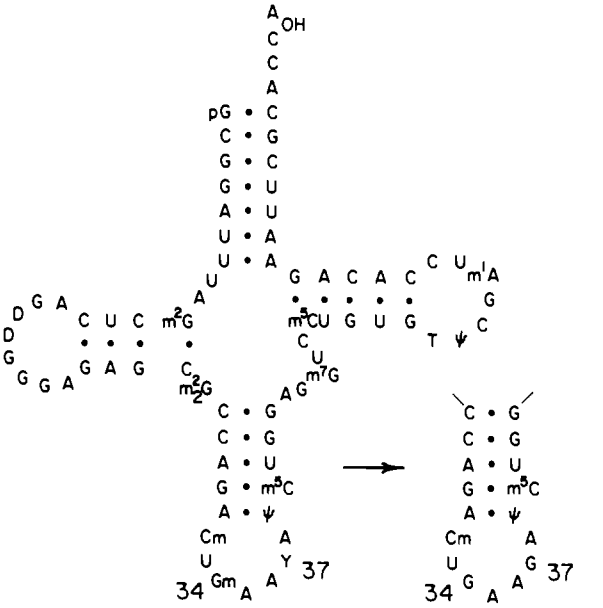

FIGURE 1: Yeast $tRNA^{Phe}$ and the anticodon arm of $tRNA^{Phe}$ substituted with GAAG.

Construction of Anticodon Substituted tRNAs. Oligonucleotides were inserted into positions 34–37 in the anticodon loop of yeast $tRNA^{Phe}$ (see Figure 1) by the six-step procedure described in detail by Bruce & Uhlenbeck (1982). First, the hypermodified Y nucleotide at position 37 is removed with acid and the chain cleaved at that point with aniline. Second, a limited digestion with pancreatic ribonuclease cleaves the chain on the 3′ side of uridine-33 and cytidine-74, resulting in two annealed half-molecules which are missing the anticodon residues 34–37 and the 3′-terminal residues 75 and 76. Third, the combined half-molecules are reacted with a tetranucleotide triphosphate acceptor and T4 RNA ligase. This results in the joining of the 3′ terminus of the tetramer to nucleotide 38 on the 5′ terminus of the 3′ half-molecule. Fourth, the substrate is treated with T4 polynucleotide kinase and ATP at pH 6.9. Under these conditions, the 3′-phosphates are removed by the 3′-phosphatase activity of the enzyme, and a phosphate is introduced onto the new 5′ terminus of the 3′ half-molecule. Fifth, the anticodon loop was resealed with a low concentration of RNA ligase. Finally, the missing 3′-terminal two nucleotides were repaired with tRNA nucleotidyltransferase.

Several slight modifications of the original procedure were introduced in order to reduce the amounts of enzymes needed by raising reactant concentrations. For the third step, the concentrations of the tRNA half-molecules, the oligonucleotide, and the ATP were increased to 30, 60, and 120 μM, respectively. The reaction was incubated for 4 h at 4 °C with 40 units/mL RNA ligase. A small aliquot of each preparative reaction was removed at this stage and analyzed on a 20% polyacrylamide denaturing gel. After the gel was stained with Stains-All, each reaction could be examined for whether the oligonucleotide had added efficiently to the 3′ half-molecule. In addition, the shifted position of the 3′ half-molecule was found in each case to correspond to that predicted by the length of the oligonucleotide acceptor.

The fourth step involving phosphate transfer with polynucleotide kinase was carried out exactly as described previously except that the concentration of tRNA halves was increased to 10 μM and ATP to 100 μM. The subsequent sealing step was also identical except that 10 units/mL RNA ligase was used. Repair of the 3′ terminus and gel purification as described previously yielded 13 different anticodon-substituted tRNAs. When the reactions were started at step 3 with 90 μg of tRNA half-molecules in each reaction, the final recovery of gel-purified tRNAs ranged from 24 to 34 μg, corresponding to an overall yield of 27–38%.

Aminoacylation. The aminoacylation reactions for determination of kinetic parameters were carried out under conditions similar to those of Thiebe & Zachau (1968). The extinction coefficient of $tRNA^{Phe}$ and of each anticodon loop substituted tRNA was assumed to be 1500 pmol/A_{260} unit. Duplicate reactions (50 μL) containing 0.03–0.3 μM tRNA, 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 25 mM KCl, 1 mM ATP, 0.01 mM L-[^{3}H]phenylalanine (11.4 Ci/mmol), 30 μg/mL bovine serum albumin, 60 mM 2-mercaptoethanol, and 0.21 unit/mL yeast phenylalanyl-tRNA synthetase were incubated at 37 °C. Aliquots (9 μL) were removed at 1, 2, 3, 5, and 10 min, spotted onto Whatman 3MM paper (1 × 2 cm), and dropped into cold 5% trichloroacetic acid. The squares were washed 5 times with 250 mL of cold trichloroacetic acid, once with 250 mL of 95% ethanol, and once with 100 mL of ether, dried, and counted in 4 g/L diphenyloxazole in toluene in a liquid scintillation counter.

It was difficult to obtain accurate aminoacylation kinetics for several of the anticodon-substituted tRNAs due to the low levels of aminoacyl-tRNA formed at low enzyme concentrations as a result of the spontaneous deacylation reaction (see Results). This problem was overcome to some extent by measuring the initial rates at four different enzyme concentrations.

Results

Characterization of Modified tRNAs. The tetranucleotide GpApApG and 12 other oligonucleotides which differ from it by a single nucleotide were synthesized enzymatically with RNA ligase and polynucleotide phosphorylase. Each oligomer was inserted into the anticodon loop of $tRNA^{Phe}$ in place of the $G_mpApApY$ sequence by the procedure reported previously (Bruce & Uhlenbeck, 1982). For a demonstration of the purity and for confirmation of the sequence of these 13 anticodon-substituted tRNAs, they were labeled at the 3′ terminus with [5′-^{32}P]pCp (Bruce & Uhlenbeck, 1978), partially digested with ribonuclease T_1, and analyzed on a 20% denaturing polyacrylamide gel (Donis-Keller et al., 1977). An autoradiogram of this gel is shown in Figure 2. The pattern of 3′-terminal labeled T_1 oligonucleotides for each of the anticodon-substituted tRNAs is very similar to a $tRNA^{Phe}$ control. The bands resulting from the six T_1 cleavages between G-42 and G-57 are identical for all 13 constructed tRNAs and $tRNA^{Phe}$. This indicates that the –CCA sequences at the 3′ termini have been properly repaired. The patterns in the upper portion of the autoradiogram (above G-30) are also identical with $tRNA^{Phe}$, indicating that the 5′ half of the molecule was correctly reattached in the substitution protocol. As expected, when GpApG or GpApApApG is inserted, the pattern of the 5′ half T_1 oligomers is shifted down or up by one nucleotide. Finally, the usually T_1-resistant anticodon loop region, G-30 to G-42, shows additional T_1 cleavage sites for each one of the anticodon-substituted tRNAs. These bands all appear at the positions predicted by the sequence of the oligonucleotide inserted. When the oligomer contained two guanosines, two bands are observed in the tRNA, and when the oligomer contained one guanosine, only one band is seen. Thus, the partial T_1 digestion pattern confirms the substitutions in the sequence expected for all 13 tRNAs.

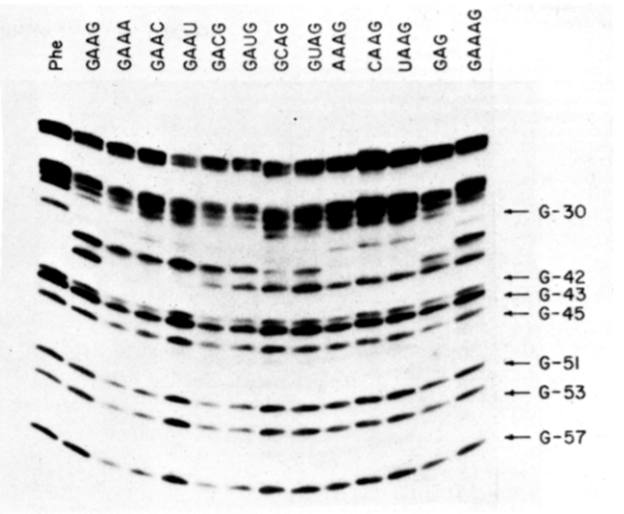

FIGURE 2: Partial ribonuclease T_1 digests of 3′-end-labeled $tRNA^{Phe}$ and 13 anticodon-substituted tRNAs. Oligonucleotides terminating with the guanosines are marked according to their position in the $tRNA^{Phe}$ sequence.

In order to be sure that the tRNAs were correctly repaired at the 3′ terminus, the 3′-labeled tRNAs were also totally digested with a mixture of ribonucleases T_1, T_2, and A and analyzed by two-dimensional chromatography on cellulose thin-layer plates (Nishimura, 1979). In each case, greater than 95% of the label was found to be AMP. Since the 3′ terminus of the starting material was a cytidine, this indicates that the CCA terminus was fully repaired.

The purity of the anticodon-substituted tRNAs is very high. Analysis of the half-molecules (residues 1–33 plus 38–74) used as starting material by gel electrophoresis showed only a very small amount of material in the position of intact $tRNA^{Phe}$ (Bruce & Uhlenbeck, 1982). In an attempt to more precisely determine the amount of tRNA-sized contaminants in a preparation of anticodon-substituted tRNA, the steps in the construction were carried out without adding an oligonucleotide acceptor before attempting to reseal the anticodon loop. When the region of the gel corresponding to tRNA-sized material from this sham reaction was cut out and eluted, less than 0.4% of the starting material was recovered. Since 20–28% of the starting material was recovered as anticodon-substituted tRNA, the amount of contaminating tRNA in each preparation must be less than 2%. It is likely that this figure overestimates the amount of contaminating tRNA since some of the material recovered in the tRNA band in the sham experiment may be tRNA resealed without an anticodon loop. This sealing reaction would not be expected to occur at a significant rate in a reaction where most of the 3′ half-molecules have an oligonucleotide attached. The aminoacylation properties of the contaminating tRNA isolated in the sham reaction were not determined due to the small amounts obtained.

Aminoacylation of Modified tRNAs. The kinetic parameters for $tRNA^{Phe}$ in the aminoacylation reaction are strongly dependent upon the ionic strength of the reaction (Bonnet & Ebel, 1972). For example, at 200 mM KCl, we have determined a K_m of 1.7 μM and a V_{max} of 3 μmol min^{-1} unit^{-1} for $tRNA^{Phe}$, similar to the values reported by Cramer & Sprinzl (1979). However, at 25 mM KCl, the K_m for $tRNA^{Phe}$ had decreased to 0.03 μM and the V_{max} increased to 80 μmol min^{-1}

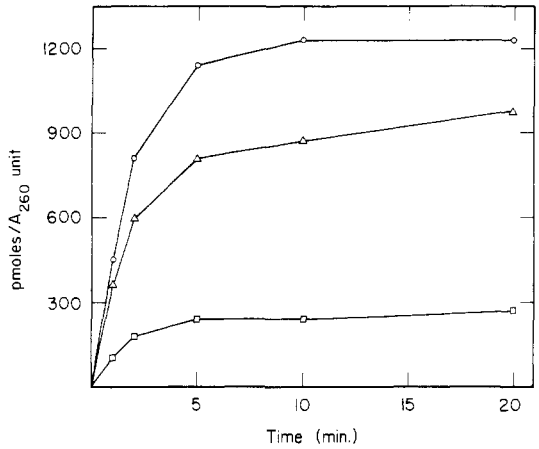

FIGURE 3: Aminoacylation of tRNAs with GpApApG (○), GpApApA (△), and ApApApG (□) inserted in the anticodon loop. Reactions were carried out with 60 nM tRNA and 0.25 unit/mL PRS.

Table I: Aminoacylation Levels of Modified tRNAs

tRNA (oligomer substituted)	position of substitution	final level (pmol/A_{260})
$tRNA^{Phe}$		1250
GAAG	37	1240
GAAA	37	980
GAAC	37	990
GAAU	37	940
GACG	36	650
GAUG	36	660
GCAG	35	460
GUAG	35	460
AAAG	34	250
CAAG	34	300
UAAG	34	300
GAAAG	+A	490
GAG	−A	310
1-33 + 38-76		80

unit^{-1}. In order to conserve material, we have chosen this lower ionic strength to perform an initial comparison of the different anticodon-substituted tRNAs.

In Figure 3 the rates of aminoacylation for three anticodon-substituted tRNAs are compared by using a relatively low PRS concentration. $tRNA^{Phe}_{GAAG}$ reacts rapidly to a level corresponding to nearly one phenylalanine per tRNA. As shown previously (Bruce & Uhlenbeck, 1982), this behavior is nearly identical with that of $tRNA^{Phe}$ and confirms the efficacy of the anticodon substitution process. $tRNA^{Phe}_{GAAA}$ reacts only slightly less well than $tRNA^{Phe}_{GAAG}$, indicating that changing position 37 in $tRNA^{Phe}$ from a guanosine to an adenosine has relatively little effect upon the aminoacylation reaction. This is consistent with the earlier observation that removal of Y-37 from $tRNA^{Phe}$ does not alter the rate or extent of reaction at low ionic strength (Thiebe et al., 1972). In contrast, $tRNA^{Phe}_{AAAG}$, which substitutes an adenosine for a guanosine in position 34, aminoacylates slowly and to a lower level under the same incubation conditions. This suggests that the guanosine in position 34 is important for active aminoacylation of $tRNA^{Phe}$ by PRS.

Similar data for the other 10 anticodon-substituted tRNAs are summarized in Table I. In each case, the tRNA is active, but the amount of aminoacyl-tRNA obtained at long incubation times is quite different. These levels are relatively well correlated with the position in the anticodon that is substituted. As the position of substitution changes from the 3′ side to the 5′ side of the anticodon loop, the level of aminoacylation decreases steadily and appears to be independent of the nature of the substitution. The replacement of G-34 with either A, C, or U results in tRNAs that aminoacylate to a similar low level. In positions 35 and 36, the same levels of aminoacylation are observed whether C or U is substituted. G was not inserted due to the difficulty of preparing the oligonucleotides GGAG and GAGG. Substitutions in position 37 have relatively little effect upon the level of aminoacylation as expected from the high levels obtained with $tRNA^{Phe}_{Y}$ (Thiebe & Zachau, 1968). Addition or deletion of an adenosine residue from the anticodon loop also results in reduced levels, and when residues 34–37 are completely removed, only a few percent of the half-molecules are aminoacylated. Taken together, these data clearly indicate that PRS is sensitive to alterations in the anticodon loop. No precise site could be identified, but alterations at the 5′ side of the loop were the most disruptive.

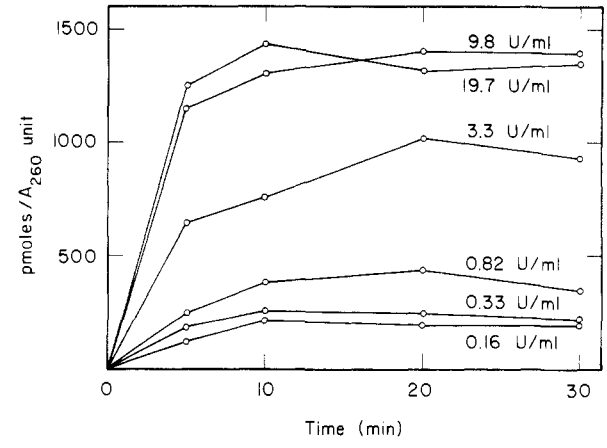

FIGURE 4: Aminoacylation of $tRNA^{Phe}_{AAAG}$ at the indicated PRS concentrations. Reactions contained 60 nM tRNA.

There are several reasons for the low level of aminoacylation observed in the anticodon-substituted tRNAs. It is unlikely that end product inhibition by pyrophosphate is the cause (Bonnet & Ebel, 1972) since the addition of 1 μg/mL pyrophosphatase failed to cause significant stimulation. It is also clear that the reactions did not become limiting in ATP due to high rates of ATP hydrolysis (Bonnet & Ebel, 1972) since experiments using [^{3}H]ATP showed that greater than 97% of the ATP remained intact at the end of the incubation period. Although it is possible that the anticodon-modified tRNAs could have an inactive conformation, pretreatment of the tRNA under conditions reported to renature $tRNA^{Phe}$ (Thiebe et al., 1972) was not successful in stimulating the reaction.

The most likely explanation for the low levels of aminoacylation of $tRNA^{Phe}_{AAAG}$ is suggested by the experiments in Figures 4 and 5. Figure 4 shows that the final level of [^{3}H]Phe-$tRNA^{Phe}_{AAAG}$ that is formed increases with increasing enzyme concentrations. At 9.8 units/mL PRS, the reaction with $tRNA^{Phe}_{AAAG}$ appears very similar to the reaction with $tRNA^{Phe}_{GAAG}$ at 0.5 unit/mL PRS in Figure 3. These data suggest that the low level of aminoacylation observed for $tRNA^{Phe}_{AAAG}$ results from a steady state between the slow nonenzymatic spontaneous deacylation of the Phe-$tRNA^{Phe}_{AAAG}$ product and the slow forward rate of reaction (Bonnet & Ebel, 1972). When higher enzyme concentrations are added, the forward rate is increased to give a higher steady-state concentration of the aminoacyl-tRNA. This view is supported by the experiment in Figure 5. When an excess of elongation factor Tu–GTP is included in the reaction mixture, both the rate and the final level of aminoacylation of $tRNA^{Phe}_{AAAG}$ are stimulated. It has been shown previously (Hopfield et al., 1976) that the rate of spontaneous deacylation of aminoacyl-tRNA is greatly reduced when it is bound to the elongation factor. Thus, the stimulation observed is a result of

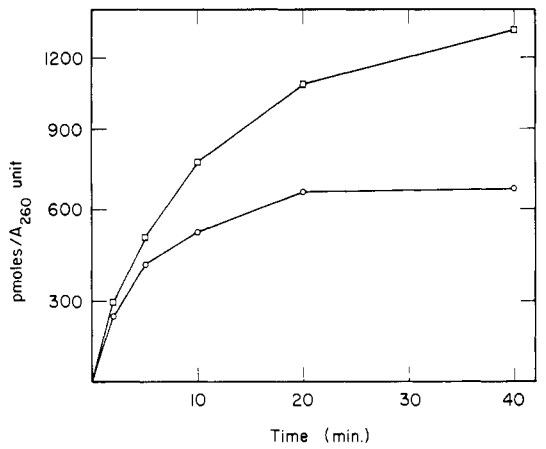

FIGURE 5: Aminoacylation of $tRNA^{Phe}_{AAAG}$ with (□) or without (○) 1.6 μM elongation factor Tu. Reactions were carried out in the normal manner with 60 nM tRNA, 0.1 mM GTP, 1.0 mM phosphoenolpyruvate, 0.1 mg/mL phosphoenolpyruvate kinase, and 16 units/mL PRS. The additional components slightly inhibit the control reaction when compared to Figure 4.

Table II: Aminoacylation Kinetics of Modified tRNAs

tRNA (oligomer substituted)	position of substitution	K_m (nM)	V_{max}[a]
$tRNA^{Phe}$		30	(1.0)
GAAG	37	43	1.5
GAAA	37	91	1.7
GAAC	37	79	1.0
GAAU	37	79	1.7
GACG	36	121	0.96
GAUG	36	71	0.98
GCAG	35	104	0.46
GUAG	35	118	0.42
AAAG	34	306	0.84
CAAG	34	250	0.77
UAAG	34	217	1.0
GAAAG	+A	165	0.69
GAG	−A	125	0.20

[a] Values of V_{max} are relative to $tRNA^{Phe}$.

reducing the rate of the competing side reaction.

The values of K_m and V_{max} determined from a Lineweaver–Burk analysis of the initial rates for $tRNA^{Phe}$ and the 13 modified tRNAs are shown in Table II. A similar pattern to that observed for aminoacylation levels in Table I can again be seen. The modifications in the anticodon loop sequence have their largest effect in increasing the K_m of the reaction. Substitutions of a nucleotide at the 3′ side of the anticodon loop (position 37) increase K_m less than 3-fold while substitutions of a nucleotide at the 5′ side of the anticodon loop (position 34) increase K_m as much as 10-fold. Substitutions at intermediate positions (35 and 36) appear to have an intermediate effect. The type of substitutions appears to be less important than the position of the substitution. With one exception, the value of V_{max} for each of the modified tRNAs is within about a factor of 2. No clear pattern emerges between the position or type of modification and the value of V_{max}.

Discussion

Schimmel (1979) has suggested that the molecular basis for the specific interaction between a tRNA and its cognate synthetase is a result of a substantial number of contacts between the protein and the tRNA spread over a fairly large area. Some of these contacts are not specific for the cognate tRNA and therefore explain the weak affinity of a synthetase for all tRNAs. Contacts which are unique for a cognate tRNA–synthetase pair presumably involve functional groups primarily located on the nucleotide rings. We have shown that replacement of nucleotides in the anticodon loop of yeast $tRNA^{Phe}$ leads to a decrease in the rate of aminoacylation with PRS due primarily to an increase in K_m. The most likely explanation of these data is that PRS interacts directly with the anticodon at some stage of the aminoacylation reaction. Since the nucleotide substitution only disrupts one of several sites of interaction between $tRNA^{Phe}$ and PRS, the specific aminoacylation reaction can still occur, but its rate is decreased due to weaker binding of the enzyme to its substrate.

Despite systematic substitution of each position in the anticodon by at least two other nucleotides, it has not been possible to identify a precise site that is responsible for the lower rate of aminoacylation. Alteration of the guanosine at position 34 to any other nucleotide produces the largest increase in K_m, suggesting that the synthetase interacts specifically with determinants on the guanosine ring. The smaller effects observed with substitution in the adjacent positions 35 and 36 may be due to disruption of the stacking on the 3′ side of the anticodon loop by the introduction of a pyrimidine. This is supported by the observation that the tRNAs with altered loop sizes ($tRNA^{Phe}_{GAG}$ and $tRNA^{Phe}_{GAAAG}$) and presumably altered loop conformations also aminoacylate poorly. An alternate possibility to a single specific contact at G-34 is that PRS contacts several nucleotides in the anticodon loop, resulting in a more complex response. The preparation of anticodon-substituted tRNAs containing more subtle modifications in the nucleotide rings could possibly resolve this issue.

Our data confirm and extend several other experiments summarized in the introduction which demonstrate an interaction between PRS and the anticodon loop of $tRNA^{Phe}$. The results obtained here also do not significantly conflict with the enzymatic dissection study of Thiebe et al. (1972), but the conclusion reached is different. Theibe et al. (1972) observed that $tRNA^{Phe}$ half-molecules (1–33 and 38–76) which deleted the anticodon gave detectable but relatively low levels of aminoacylation under standard conditions. They concluded that these low levels were the result of inefficient annealing of the half-molecules, and therefore the anticodon did not significantly contribute to the tRNA–synthetase interaction. In this work, the low levels of aminoacylation encountered with the anticodon-substituted $tRNA^{Phe}$ were found to be a result of a reduction in the forward rate of reaction combined with the constant rate of spontaneous hydrolysis of aminoacyl-tRNA. It is likely that this explanation is also correct for the half-molecules. Thus, it would appear that the anticodon does contribute significantly to the interaction between $tRNA^{Phe}$ and PRS.

Since each tRNA must differ from tRNAs specific for other amino acids in its anticodon sequence, it is not surprising that the anticodon is a site for a specific interaction between tRNA and its synthetase. The situation discussed here with yeast PRS has also been reported for several other synthetases from *E. coli*. However, the degree to which the contact in the anticodon region contributes to the total tRNA–synthetase interaction varies considerably. Disturbing the anticodon structure of *E. coli* $tRNA_f^{Met}$ (Schulman & Pelka, 1977) or $tRNA^{Gly}$ (Carbon & Squires, 1971) causes a nearly 1000-fold decrease in the rate of aminoacylation. In contrast, altering the sequence of *E. coli* $tRNA^{Trp}$ has a more modest 10-fold effect on the rate, similar to what is seen here with $tRNA^{Phe}$. However, the importance of the anticodon contact to the specificity of the interaction cannot be deduced from its absolute magnitude. The single nucleotide change in $tRNA^{Trp}$

causes it to be aminoacylated by the glutamine synthetase at significant rates (Yarus et al., 1977). Thus, in that case much of the specificity of the interaction lies in the anticodon contact.

Studies on the kinetics of heterologous misacylation of a variety of tRNAs by PRS give results which are significantly different from those obtained here. Roe et al. (1973) measured misacylation of seven different *E. coli* tRNAs with yeast PRS and found that the primary cause for decreased aminoacylation rates was a decreased value of V_{max}. These results were consistent with those of Ebel et al. (1973) with *E. coli* valyl-tRNA synthetase and led to the suggestion that although discrimination of the correct tRNA by the synthetase can act both at the level of K_m and of V_{max}, the latter parameter was the more effective. Although the explanation for such a discrimination mechanism was not clear, Krauss et al. (1976) proposed that tRNAs which are unable to undergo a required conformational change will have a low V_{max}. Since our results show that anticodon-substituted tRNAs aminoacylate poorly, almost entirely as a result of an increased K_m, it is possible that discrimination might occur by different means at different parts of the molecule.

By comparing tRNA sequences which could be acylated by yeast PRS, Roe et al. (1973) used their data to deduce two regions on tRNA which contained the minimal essential contacts for the proper interaction with PRS. These regions included nine nucleotides in the dihydrouridine stem and nucleotide A-73. Since several tRNAs with different anticodons could be misacylated by PRS, the anticodon region was not considered to be essential. However, of the 11 tRNAs Roe et al. examined, those 7 with altered anticodons misacylated with very high K_m and very low V_{max} values, suggesting that the anticodon region is quite important. In addition, Feldman & Zachau (1977) noted that yeast $tRNA^{Met}$, which contains the essential regions deduced by Roe et al. (1973) but a very different anticodon sequence than $tRNA^{Phe}$, is not active with PRS. Finally, $tRNA^{Phe}$ from *S. pombe* has the same anticodon as $tRNA^{Phe}$ from *S. cervacia* but a significantly different dihydrouridine stem and aminoacylates normally with PRS (McCutchan et al., 1978). These data as well as those obtained here can be reconciled by proposing that PRS interacts with $tRNA^{Phe}$ at specific functional groups within the two areas proposed by Roe et al. (1973), at the 5′ side of the anticodon region as discussed here and very possibly at one or more other sites. If one of these contacts is disrupted by a chemical modification or a nucleotide substitution, the rate of aminoacylation will decrease to an extent depending upon the nature and the position of the modification. The situation is more complex when one considers the interaction of other tRNAs with PRS. In this case, while several of the functional groups involved in specific contacts with PRS may be present and properly oriented, other groups may be located at positions that interfere with PRS binding. Since each tRNA can have a different number of such interfering groups, it is difficult to accurately deduce the specific contacts from comparing aminoacylation rates of tRNAs that differ substantially in sequence. This point emphasizes the importance of using relatively modest changes in tRNA molecules to deduce contact points with proteins. The nucleotide substitution procedure described here provides one such approach.

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