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Specific Substitution into the Anticodon Loop of Yeast Tyrosine Transfer RNA†

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ABSTRACT: The aminoacylation kinetics of 19 different variants of yeast $tRNA^{Tyr}$ with nucleotide substitutions in positions 33–35 were determined. Substitution of the conserved uridine-33 does not alter the rate of aminoacylation. However, substitution of the anticodon position 34 or position 35 reduces K_m from 2- to 10-fold and V_{max} as much as 2-fold, depending on the nucleotide inserted. The ochre and amber suppressor $tRNAs^{Tyr}$ both showed about a 7-fold reduction in V_{max}/K_m. Data from $tRNA^{Tyr}$ with different modified nucleotides at position 35 suggest that specific hydrogen bonds form between the synthetase and both the N1 and N3 hydrogens of Ψ-35. The effect of simultaneous substitutions at positions 34 and 35 can be predicted reasonably well by combining the effects of single substitutions. These data suggest that yeast tyrosyl-tRNA synthetase interacts with positions 34 and 35 of the anticodon of $tRNA^{Tyr}$ and opens the possibility that nonsense suppressor efficiency may be mediated by the level of aminoacylation.

The anticodon loop of many tRNAs[1] form part of the recognition site for their cognate aminoacyl-tRNA synthetase (Kisselev, 1985). In the case of the yeast tyrosine tRNA, the available data appear contradictory. On one hand, two suppressor tRNAs derived from the $tRNA^{Tyr}$ by single-base substitutions of the wobble anticodon nucleotide G-35 still insert tyrosine into protein, suggesting that the substitution does not interfere with their ability to aminoacylate (Piper et al., 1976; Goodman et al., 1976). In addition, removal of the anticodon nucleotides 34–36 from the closely related *Torula utilis* $tRNA^{Tyr}$ does not eliminate aminoacylation by yeast tyrosoyl-tRNA synthetase (Hashimoto et al., 1972). On the other hand, the yeast $tRNA^{Tyr}$ precursor containing a 15-nucleotide intron in the anticodon loop does not aminoacylate (Valenzuela et al., 1980), despite the fact that the structure of the remainder of the tRNA appears to be normal (Swerdlow & Guthrie, 1984). Furthermore, in a previous paper, we

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[1] Abbreviations: $tRNA^{Tyr}$, yeast tyrosine transfer RNA; pNp, nucleoside 3′,5′-bisphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-(2-hydroxylmethyl)-1,3-propanediol; TCA, trichloroacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

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Table I: Construction of $tRNA^{Tyr}$ Variants

type of variant	5′ half	donor oligomer	3′ half
position 33	1–32	pNpGp	35–76
position 34	1–32	pUpNp	35–76
position 35	1–34	pNp	36–74
positions 34 and 35	1–32	pUNNp or pUNp + pNp	36–74

developed a protocol for substituting nucleotides into the anticodon of yeast $tRNA^{Tyr}$ and demonstrated in a qualitative fashion that three of the substituted $tRNAs^{Tyr}$ did not aminoacylate as well as the starting material (Bare & Uhlenbeck, 1985).

In order to clarify the role of the anticodon of yeast $tRNA^{Tyr}$ in the aminoacylation reaction, we have developed an improved anticodon loop substitution protocol and used it to prepare a set of $tRNAs^{Tyr}$ that contains several different substitutions at each of three anticodon loop positions. Analysis of the steady-state kinetics of the aminoacylation reaction under several reaction conditions permits the identification of those parts of the anticodon loop that are necessary for proper interaction with the synthetase.

Materials and Methods

Enzymes. Polynucleotide kinase and polynucleotide kinase lacking the 3′-phosphatase were purified from T4- and *pseT1* T4-infected *Escherichia coli*, respectively (Soltis & Uhlenbeck, 1982). Primer-dependent polynucleotide phosphorylase was purified from *Micrococcus luteus* (Klee, 1971). Yeast tRNA nucleotidyl transferase was a gift from A. G. Bruce. RNA ligase purified from a recombinant *E. coli* (Rand & Gait, 1984) was a gift of R. I. Gumport. Bacterial alkaline phosphatase (BAPF) was purchased from Worthington Biochemicals. Calf intestinal alkaline phosphatase, ribonuclease A, ribonuclease T_1, and bovine brain nucleoside 2′,3′-cyclic phosphate 2′-nucleotidohydrolase (EC 3.1.4.37) were purchased from Sigma Chemical Co. Yeast tyrosyl-tRNA synthetase was purified to a specific activity of 280 units/mg by using the first three steps of the procedure of Faulhammer & Cramer (1977).

Nucleotides. The 3′(2′),5′-bisphosphates of 3-methyluridine (m^3U), 3-deazauridine (3deazaU), dehydrouridine (D), 5-fluorouridine (f^5U) and pseudouridine (Ψ) were made by phosphorylation of the corresponding nucleosides with pyrophosphoryl chloride (Barrio et al., 1978) and were the gift of W. Wittenberg. pUp, pCp, and pAp were purchased from P-L Biochemicals. UpGp and ApGp were synthesized as described in Bare and Uhlenbeck (1985). CpGp was isolated from a ribonuclease T_1 digest of poly(CG). UpCp, UpAp, UpCpUp, pUpUp, and pUpUpUp were made by periodate oxidation and β elimination of UpCpU, UpApC, UpCpUpU, pUpUpU, and pUpUpUpU, respectively. UpCpCp was made by the addition of pCp to GpUpC followed by ribonuclease T_1 digestion. Oligonucleotides with 3′-phosphates were 5′-phosphorylated by using *pseT1* polynucleotide kinase (Cameron et al., 1978). Yeast $tRNA^{Tyr}$ was purified as described by Maxwell et al. (1968).

Construction of tRNA Variants. The replacement of nucleotides at positions 33–35 of yeast $tRNA^{Tyr}$ was carried out using one of three similar protocols depending upon the substitution desired (Table I). Figure 1 shows the general strategy for all three protocols and the individual steps of the protocol used to replace Ψ-35 with different nucleotides. The overall procedure involves subjecting $tRNA^{Tyr}$ either to partial ribonuclease T_1 digestion to give fragments 1–34 and 35–76 or to partial ribonuclease A digestion to give fragments 1–32 and 36–74. After purification of the fragments by gel electrophoresis, the 5′ half-molecules (1–32 or 1–34) are dephosphorylated with alkaline phosphatase and the 3′ half-molecules (35–76 or 36–74) are treated with polynucleotide kinase to remove their 3′-terminal phosphate and add a 5′-terminal phosphate. A short donor molecule with 5′- and 3′-phosphates is then added to the 3′ terminus of the 5′ half-molecule using T4 RNA ligase. The product of the ligase reaction is then treated with polynucleotide kinase to remove the 3′-terminal phosphate and introduce a 5′-terminal phosphate, and the fragment is purified by gel electrophoresis. This modified 5′ half-molecule is then annealed to the appropriate 3′ half-molecule (Table I), and the anticodon loop is sealed with RNA ligase. After the 3′ terminus is repaired with tRNA nucleotidyl transferase, if necessary, the variant $tRNA^{Tyr}$ is purified by gel electrophoresis. Experimental details for these steps follow.

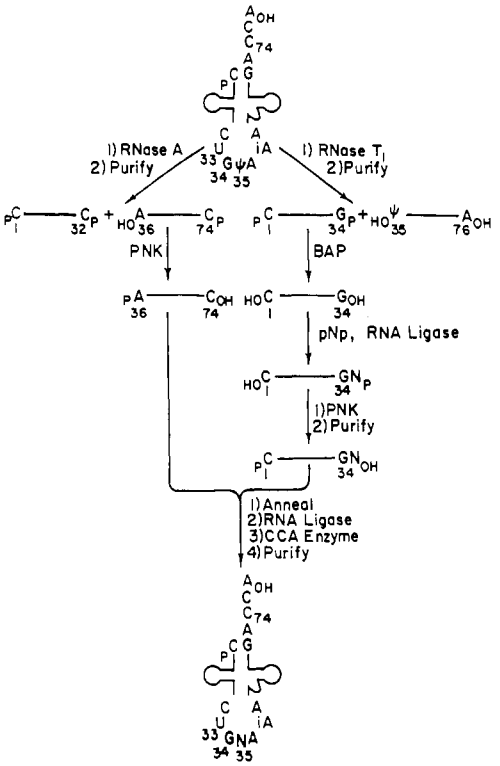

FIGURE 1: Steps used to substitute residue 35 in yeast $tRNA^{Tyr}$. Abbreviations: RNase A, pancreatic ribonuclease; RNase T_1, ribonuclease T_1; PNK, T4 polynucleotide kinase; BAP, bacterial alkaline phosphatase; CCA enzyme, tRNA nucleotidyl transferase; pNp, nucleoside 3′,5′-bisphosphate.

Partial ribonuclease A or T_1 digestions of $tRNA^{Tyr}$ were carried out in reactions containing 0.5 mg/mL $tRNA^{Tyr}$, 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM KCl, and 20 μg/mL ribonuclease. Incubation was at 0 °C for 2–2.5 h. For the ribonuclease A reaction, cleavage at the 3′ terminus to remove C-75 and A-76 was complete in minutes, the cleavages after U-33 and Ψ-35 were somewhat slower, and nearly 2 h was required to complete the cleavage at C-32. For the ribonuclease T_1 reaction the cleavage at G-34 progressed steadily with only slight hydrolysis in the D-loop toward the end of the incubation time. The ribonuclease digestions were terminated by adding 15 μL of diethyl pyrocarbonate/mL reaction, vortexing for 1 min, and precipitating with ethanol. The half-molecules were purified by gel electrophoresis as described previously (Wittenberg & Uhlenbeck, 1985).

The purified 5′ half-molecules (1–32 or 1–34) were dephosphorylated in a reaction containing 42 μM half molecule, 200 mM Hepes, pH 6.8, 30 mM $MgCl_2$, 1.5 mM DTT, 80

μg/mL alkaline phosphatase, and 5 units/mL bovine brain nucleotidohydrolase. After incubation for 2 h at 37 °C, the reaction was phenol-extracted and ether-extracted, and the half-molecules were recovered by ethanol precipitation. The essentially quantitative removal of the 3′- and 5′-phosphates could be followed by a decrease in eleectrophoretic mobility approximately equal to the addition of one nucleotide. However, if the bovine brain nucleotidohydrolase was omitted from reaction, 10–20% of the half-molecules were resistant to dephosphorylation by alkaline phosphatase despite prolonged incubation. Presumably this fraction of molecules contain a terminal 2′,3′-cyclic phosphate as a result of the partial nuclease digestion. The inclusion of the bovine brain nucleotidohydrolase, which opens 2′,3′-cyclic phosphates to 2′-phosphates (Drummond et al., 1962), solved this problem.

The addition of 5′,3′-phosphorylated donor molecules (monomers, dimers, or trimers) to the dephosphorylated 5′ half-molecule were carried out in a reaction containing 3 μM 5′ half-molecule, 225 μM ATP, 100–200 μM donor, 50 mM Hepes pH 8.3, 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, and 75 μg/mL RNA ligase, and the reaction was incubated for 4 h at 25 °C. Analysis of the reactions by gel electrophoresis revealed products migrating slower than the half-molecule when dimer or trimer donors were used. However, no mobility shift occurs when a monomer is added unless the reaction is dephosphorylated prior to gel electrophoresis. For the above conditions, reaction yields were usually greater than 90%. Less RNA ligase could be used with more reactive donors (England & Uhlenbeck, 1978). In the case where two sequential additions of donors to the half-molecule were used, the first addition reaction was terminated by heating to 65 °C for 5 min and calf intestine alkaline phosphatase was added to 50 μg/mL and incubated for 2 h at 37 °C. The dephosphorylation reaction was terminated by the addition of ethylenediaminetetraacetic acid to 10 mM and sodium dodecyl sulfate to 0.1% and heating to 100 °C for 2 min. After a further incubation at 65 °C for 15 min, the dephosphorylated product was ethanol-precipitated twice prior to the second RNA ligase reaction.

Removal of the 3′-terminal phosphate and reintroduction of the 5′-terminal phosphate to the modified 5′ half-molecules was carried out by incubating 10 μM 5′ half-molecule, 100 μM ATP, 50 mM Hepes, pH 6.8, 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, and 100 units/mL T4 polynucleotide kinase for 2 h at 25 °C. The product was ethanol-precipitated and purified by gel electrophoresis. The same protocol was used on the isolated 3′ half-molecules to prepare them for joining to the modified 5′ half-molecules. In this case the gel purification of the product was omitted. When the 36–74 half-molecule was used, 5 units/mL bovine brain nucleotidohydrolase was included in the reaction to open any 2′,3′-cyclic phosphate at the 3′ terminus.

To prepare the substituted tRNA, the two half-molecules were annealed in a 700-μL reaction containing 1.5 μM 5′ half-molecule, 1.6 μM 3′ half-molecule, 20 μM ATP, 50 mM Hepes, pH 8.3, 20 mM $MgCl_2$, and 3.3 mM DTT, and heated to 65 °C for 5 min followed by 15 min at 37 °C. The anticodon loop is sealed by adding RNA ligase to 15 μg/mL and incubating for 1 h at 25 °C. The reaction is stopped by heating to 65 °C for 5 min. If annealing is incomplete, the half-molecules circularize, causing them to migrate faster on the gels. The slight excess of 3′ half-molecule is to ensure optimal use of the more valuable 5′ half-molecule. For reactions using the 3′ half-molecule 36–74, the repair of the CCA terminus was carried out as reported previously (Bare & Uhlenbeck, 1985). The anticodon modified tRNAs were purified by gel electrophoresis as previously described (Bruce & Uhlenbeck, 1982a).

The correct insertion of oligomers into the anticodon of $tRNA^{Tyr}$ was verified by including [γ-^{32}P]ATP in the phosphorylation of the 3′ half-molecule. When the internally ^{32}P-labeled $tRNA^{Tyr}$ was hydrolyzed to 3′-monophosphates, the only radioactive monomer corresponded to the 3′ end of the inserted donor (Wittenberg & Uhlenbeck, 1985).

This procedure differs from the one previously described for $tRNA^{Tyr}$ (Bare & Uhlenbeck, 1985) in that an oligomer donor is added to an isolated half-molecule instead of an oligomer acceptor added to a "gapped" tRNA. A major advantage of this procedure for $tRNA^{Tyr}$ is that the overall yields were substantially higher. The reaction yields at each step were analyzed by gel electrophoresis and usually found to be greater than 90%. Losses primarily from the two gel purification steps resulted in a 15–35% overall yield starting from isolated half-molecules. This is about 5 times higher than for the method previously described. In addition, the present method also allows for the facile insertion of modified nucleotides into the anticodon using nucleoside 5′,3′-bisphosphates. Several other groups have used a similar strategy to prepare other anticodon-substituted tRNAs (Ohtsuka et al., 1983; Wittenberg & Uhlenbeck, 1985; Vacher et al., 1984; Shulman & Pelka, 1983a).

Aminoacylation Kinetics. Three different reaction buffers were used to evaluate the ability of anticodon-substituted tRNAs. Buffer A contained 30 mM Hepes–KOH, pH 7.5, 15 mM $MgCl_2$, 55 mM KCl, 2 mM DTT, 2 mM ATP, 100 μg/mL BSA, and 10 μM [^{3}H]tyrosine (43–51 Ci/mmol). Buffer B contained 30 mM Hepes–KOH, pH 7.5, 1.1 mM $MgCl_2$, 55 mM KCl, 2 mM DTT, 1 mM ATP, 1 mM spermine, 100 μg/mL BSA, and 10 μM [^{3}H]tyrosine (43–51 Ci/mmol). Buffer C contained 30 mM Hepes–KOH, pH 7.5, 2.5 mM $Mg(OAc)_2$, 1.5 mM ATP, 140 mM KOAc, 2 mM DTT, 1 mM spermine, 100 μg/mL BSA, and 20 μM [^{3}H]-tyrosine (43–51 Ci/mmol). Six different tRNA concentrations were chosen for each modified tRNA so as to span the K_m. Tyrosyl-tRNA synthetase was diluted to 0.25 unit/mL in buffer A containing 10% glycerol and no ATP or tyrosine, and stored at 0 °C prior to use. A typical reaction involved mixing concentrated buffer stock and tRNA to a total voume of 59 μL and preincubating for 8 min at 37 °C. Then 5 μL of enzyme was added and five 10-μL aliquots were taken at 15-s intervals and spotted onto dry 3MM paper presoaked in 2 mM tyrosine and 10% TCA. The papers were washed once in 1 mM tyrosine and 5% TCA, four times in 5% TCA, once in 95% ethanol, and once in ether. After drying, the papers were counted in a liquid scintillation counter in 0.4% diphenyloxazole in toluene. Backgrounds were subtracted and initial rates calculated by using least-squares analysis. K_m and V_{max} were determined from the initial rates by using the method of Eadie–Hofstee (Hofstee, 1959). Each group of variants was compared to a $tRNA^{Tyr}$ standard to ensure reproducibility.

Results

The consequences of substituting each of the nucleotides in positions 33–35 of yeast $tRNA^{Tyr}$ on the kinetics of aminoacylation with purified yeast tyrosyl-tRNA synthetase are summarized in Table II. In the buffer conditions used (buffer A) $tRNA^{Tyr}$ has a K_m of 54 nM and V_{max} of 280 nmol min^{-1} mg^{-1}. Since the enzyme preparation was not homogeneous, variant tRNAs will be compared by using an arbitrarily chosen V_{max} = 100 for $tRNA^{Tyr}$. Two control tRNAs were prepared where UpG was reinserted into positions 33 and 34 and Ψ was

Table II: Aminoacylation Kinetics of Singly Substituted $tRNAs^{Tyr}$

	K_m (nM)	V_{max}	V_m/K_m
$tRNA^{Tyr}$	54	(100)	1.9
U-33 variants			
U (control)	52	98	1.9
A	46	90	2.0
C	50	85	1.7
G-34 variants			
G (control)	52	98	1.9
U	270	71	0.26
C	230	48	0.21
A	550	80	0.15
Ψ-35 variants			
Ψ (control)	50	100	2.0
U	96	90	0.94
f^5U	150	120	0.80
D	180	120	0.67
m^3U	430	100	0.23
3deazaU	440	100	0.23
C	340	72	0.21
A	430	57	0.13

Table III: Aminoacylation Kinetics of Doubly Substituted $tRNAs^{Tyr}$

	K_m (nM)	V_m	V_m/K_m	calcd V_m/K_m
$tRNA^{Tyr}$	54	(100)	1.9	
C-34, U-35	530	27	0.051	0.10
C-34, C-35	2500	18	0.0072	0.023
U-34, U-35	1100	51	0.046	0.13
U-34, C-35	2500	18	0.0072	0.029

Table IV: Aminoacylation Kinetics in Different Buffers

	buffer A		buffer B		buffer C	
tRNA	K_m (nM)	V_{max}	K_m (nM)	V_{max}	K_m (nM)	V_{max}
$tRNA^{Tyr}$	54	(100)	160	200	490	610
A-33	46	100	170	230	440	660
U-34	270	70	1500	96	2800	280
U-35	100	100	520	160	1500	660
C-35	230	50	1300	54	4100	200

reinserted into position 35. Since both controls showed values of K_m and V_{max} virtually identical with those of $tRNA^{Tyr}$, it is clear that the substitution process did not alter the activity of the tRNA.

Each of the three positions in the anticodon loop showed different sensitivities to substitution. The highly conserved uridine at position 33 can be substituted by a C or an A without significant change in K_m or V_{max}. In contrast, substitution of either G-34 or Ψ-35 by any other nucleotide reduces the rate of aminoacylation substantially. In both cases, the change is primarily in the value of K_m, which can increase by as much as 10-fold, depending upon the nature of the substitution. Smaller changes in V_{max} are observed in some cases. Therefore, only the two anticodon nucleotides G-34 and Ψ-35 are needed for the optimal rate of aminoacylation.

In an attempt to identify which functional groups on Ψ-35 were important for the specific interaction with the synthetase, the aminoacylation kinetics of several variant $tRNAs^{Tyr}$ with modified pyrimidines at position 35 were determined. Although the set of pyrimidines were modified uridines rather than modified pseudouridines, some interesting conclusions can be made. The Ψ-35 variants can be categorized into two groups, according to the extent which V_{max}/K_m is reduced. V_{max}/K_m is 2- to 3-fold lower for the U, f^5U, and D variants and 8- to 10-fold lower for the C, m^3U, and 3deazaU variants. A simple explanation of these data is that both heterocyclic NH groups of Ψ-35 are needed for optimal contact with the synthetase. All three members of the first group of variants have modifications that could disrupt a contact between the protein and the N1 position of Ψ. All three members of the second group could disrupt both the N1 and N3 positions. Since a 2- to 3-fold decrease in V_{max}/K_m accompanies the disruption of a single hydrogen bond in the binding of tyrosyl-adenylate to tyrosyl-tRNA synthetase (Fersht et al., 1985), it is possible that the two NH groups of Ψ-35 are acting as hydrogen-bond donors. Of course, other parts of the Ψ ring may be interacting with the enzyme as well. However, it is unlikely that the C2 keto of Ψ is involved since the V_{max}/K_m value of m^3U, which has the keto function, is very similar to those of C and 3deazaU, which do not.

It should be emphasized, however, that the explanation of the kinetic data in terms of the inactivation of functional groups on the nucleotide assumes that the conformation of a variant is essentially identical with the parental compound. Thus other explanations of the data remain possible. For example, Ψ-39 of yeast $tRNA^{Phe}$ has been shown to be in the syn conformation (Roy et al., 1982). It is possible that Ψ-35 in $tRNA^{Tyr}$ may need to be in the syn conformation for proper interaction with the synthetase, and a portion of the V_{max}/K_m changes reflects the free energy required to change the U analogues from the anti to the syn configuration. The preparation of modified Ψ analogues at position 35 may help distinguish between these possibilities.

Since substitution of nucleotides in both positions 34 and 35 of $tRNA^{Tyr}$ led to a decreased rate of aminoacylation, it was of interest to determine whether the effects were independent. The change in binding free energy, $\Delta\Delta G_b$, caused by a single nucleotide substitution, can be calculated by using the V_{max}/K_m values of yeast $tRNA^{Tyr}$ and each variant (Fersht, 1977). By adding the $\Delta\Delta G_b$'s of a position 34 and a position 35 variant, $\Delta\Delta G_b$ and V_{max}/K_m can be calculated for each double variant, assuming that the $\Delta\Delta G_b$'s of the single variants are independent. Table III gives the kinetic properties of several double variants, and the experimental V_{max}/K_m values are compared with those calculated from the appropriate two single variants. In every case the double variant aminoacylated less well than either corresponding variant. It appears that the contributions from each position are roughly independent since the experimental V_{max}/K_m is only slightly less than expected if each position contributed to $\Delta\Delta G_b$ independently.

In order to reduce the K_m of the valuable $tRNA^{Tyr}$ variants, the aminoacylation kinetics determined in the previous sections used a low ionic strength buffer and saturating concentrations of tyrosine and ATP. Since buffer conditions can greatly affect the specificity of aminoacylation, it was of interest to determine whether the results obtained by using buffer A were modified under different buffer conditions. Buffer B is very similar to the conditions used by Loftfield et al. (1981) to maximize the accuracy of yeast phenylalanyl-tRNA synthetase. If spermine is included and the ATP and magnesium concentrations are reduced to a more physiological level, the V_{max} of the cognate tRNA is stimulated with respect to noncognate tRNAs. Buffer C is similar to one used by Atkins et al. (1975) for accurate in vitro protein synthesis. It also contains spermine and low magnesium and ATP concentrations, but has a more physiological concentratrion of KCl. Potassium ion has been shown to stimulate tyrosyl-tRNA synthetase (Beikirch et al., 1972). The kinetic data comparing $tRNA^{Tyr}$ and four anticodon variants in all three buffers are given in Table IV. Both the K_m and the V_{max} of $tRNA^{Tyr}$ are increased in buffers B and C. However, the K_m and V_{max} values of the variants are also changed so the conclusions of the previous sections are unaltered. Substituting U-33 has no effect on the kinetic parameters while substituting G-34 or Ψ-35 increases K_m

substantially and slightly reduces V_{max}.

Discussion

The positions in the anticodon loop of yeast $tRNA^{Tyr}$ found to be important for a maximal rate of aminoacylation appear to be the same as those previously identified for yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{fMet}$ in similar experiments (Bruce & Uhlenbeck, 1982b; Wittenberg & Uhlenbeck, 1985; Schulman & Pelka, 1983a). The conserved uridine-33 can be modified to any other nucleotide without affecting aminoacylation kinetics, but the anticodon nucleotides must be unchanged for an optimal rate of reaction. Since the structures of the three anticodon loops are likely to be quite similar, it appears that all three enzymes contact this part of the tRNA in a similar fashion. If, as proposed by Rich and Schimmel (1977), the enzymes interact with their cognate tRNAs on the "inside" of the L-shaped tertiary structure, the functional groups of the anticodon nucleotides will be available for contacts with the protein whereas U-33 is on the opposite side of the molecule. Despite this structural similarity, it is clear that the importance of the anticodon loop contacts to the K_m and V_{max} of aminoacylation differs substantially for the three tRNA-synthetase interactions. In the case of $tRNA^{Tyr}$ and $tRNA^{Phe}$, changing nucleotides in either position 34 or 35 reduces V_{max}/K_m by 10-fold or less whereas the corresponding changes in $tRNA^{fMet}$ reduce V_{max}/K_m by as much as 10^6-fold (Schulman & Pelka, 1983b). Thus, the anticodon contacts are much less important for the tyrosine and phenylalanine enzymes.

The tyrosine and phenylalanine synthetases show an identical response when G-34 of their cognate tRNAs is changed to other nucleotides. In each case, V_{max}/K_m decreases in the order G > U > C > A, by nearly the same magnitude. This raises the possibility that the two enzymes interact with their cognate tRNAs in exactly the same way at this position. If the same situation occurs at position 36 where both tRNAs have an adenosine, it would mean that the tyrosine and phenylalanine enzymes discriminate between each other's anticodon loops primarily by contacts at position 35. This view is supported by the fact that $tRNA^{Tyr}$ (A-35) is misacylated by the phenylalanine synthetase (Bare & Uhlenbeck, 1985) and $tRNA^{Phe}$ with a U at 35 can misacylate with tyrosyl-tRNA synthetase (Bare, unpublished experiments). Since the misacylation rates are low, additional residues outside the anticodon must also be important in discriminating between the two tRNAs.

It is interesting to note that $tRNA^{Tyr}$ (A-34) aminoacylates the poorest of all variants tested. Among more than 300 available tRNA sequences, an adenosine has never been found at position 34. Perhaps the A-34 tRNA forms an unusual anticodon loop conformation (Balasubramananian & Seetharamulu, 1981), which might interfere with the synthetase interaction.

The relatively small change in V_{max}/K_m when Ψ-35 of $tRNA^{Tyr}$ is changed to an A suggests that the contact must be limited to a few hydrogen bonds. The attempt to identify the functional groups on the pseudouridine ring that might act as hydrogen-bond donors or acceptors was not entirely conclusive but suggests that both ring NH groups may act as donors. Several other modified pseudouridine analogues would have to be tested to support this model.

Three of the tRNAs produced in this work are expected to be nonsense suppressor tRNAs. $tRNA^{Tyr}$ (C-35) has a sequence identical with that of the yeast SUP 5 amber suppressor tRNA (Piper et al., 1976) $tRNA^{Tyr}$ (U-35) is identical with the yeast SUP 6 ochre suppressor tRNA (Johnson & Abelson, 1983) except that the uridine ring is not modified to 5-[(methoxycarbonyl)methyl]-2-thiouridine. Finally, $tRNA^{Tyr}$ (U-34, C-35) should be a UGA suppressor tRNA for which no natural counterpart has been found. When precharged with tyrosine, all three of these tRNAs are able to suppress the appropriate termination codon in an eukaryotic in vitro suppression assay (Atkins et al., 1975) with an activity similar to that of their natural counterparts (G. Bruce, personal communication). Since all three of the synthetic suppressor $tRNAs^{Tyr}$ show a reduced rate of aminoacylation with yeast tyrosol synthetase, the same is likely to be the case for the SUP 5 and SUP 6 suppressor tRNAs. In two other cases where the aminoacylation kinetics of purified suppressor tRNAs have been carried out, *E. coli* Su^{+7} $tRNA^{Trp}$ shows a reduced rate of aminoacylation (Yarus et al., 1977) and *E. coli* Su^{+3} $tRNA^{Tyr}$ aminoacylates normally (Abelson et al., 1970). Thus, no simple correlation can be made between the existence of an active suppressor tRNA and the interaction of the corresponding synthetase with anticodon nucleotides.

The absence of modifications of the anticodon loop nucleotides of suppressor tRNAs often reduces the efficiency of suppression (Colby et al., 1976; Johnson & Abelson, 1983; Laten et al., 1980; Heyer et al., 1984). While it is generally assumed that the modification is necessary for an optimal interaction of the suppressor tRNA with the ribosome, it also seems possible that the undermodified tRNA may be incompletely aminoacylated. Johnson and Abelson (1983) have shown that when the intron of the yeast SUP 6 gene is removed, the suppressor tRNA does not have the Ψ modification at position 35. While this tRNA is fully active as a suppressor when the gene is present on the multicopy plasmid, it is much less active when the gene is present in a single copy. Since we show here that the replacement of Ψ-35 by a U increases K_m about 4-fold, the reduced suppressor efficiency of the tRNA from the gene single copy may simply be a consequence of incomplete aminoacylation. When more gene copies are present, the intracellular suppressor tRNA concentration is high enough to overcome the increased K_m. A possible way to test this interpretation in vivo is to see whether the overproduction of tyrosyl synthetase would increase the suppressor efficiency of the tRNA from the single copy gene.

The results in this work do not significantly conflict with those of Hashimoto et al. (1972), but the conclusion reached is different. Both the *T. utilis* $tRNA^{Tyr}$ missing anticodon loop residues that they studied and the anticodon substituted $tRNAs^{Tyr}$ studied here could be aminoacylated with yeast tyrosyl synthetase although the level of aminoacylation was somewhat lower than the corresponding unaltered tRNAs. We have shown that this lower level is the result of a reduced forward rate of reaction combined with the constant rate of spontaneous deacylation (Bonnet & Ebel, 1972). Hashimoto et al. (1972) conclude that since the modified tRNA can still aminoacylate, the recognition site has not been disturbed. In contrast, we believe that tRNA-synthetase recognition is achieved by the formation of a substantial number of contacts between the surface of the two macromolecules and that a reduction of the forward reaction rate indicates that part of the recognition site has been modified. It is difficult to predict the extent that the reaction rate will be reduced when a given contact is altered. This will depend upon the contribution of the contact to the entire interaction as well as the change that has been made. In the case of changes in the $tRNA^{Tyr}$ anticodon loop, the diminution of rate is never more than about 10-fold. However, the contribution of this contact to the overall recognition process remains unclear until other contacts

can be evaluated. If the situation is similar to that in several DNA binding proteins that have a large binding site (Goeddel et al., 1978) the contribution of a given base pair to the overall binding constant is rarely more than 10-fold. If this is the case for the tyrosyl-tRNA synthetase interaction most nucleotide changes will not entirely eliminate the ability of the tRNA to aminoacylate.

In summary, in spite of earlier evidence to the contrary, yeast tyrosyl-tRNA synthetase resembles many other tRNA synthetases in forming contacts with the anticodon of its cognate tRNA. As originally pointed out by Kisselev and Fralova (1964), the single-stranded anticodon residues are ideal candidates for specific contacts that can distinguish between tRNAs. It seems quite possible that all tRNA synthetases will form contacts in this region. In this regard the serine synthetases, which must inspect isoacceptor tRNAs with very different anticodons, would be especially interesting to study.

Registry No. G, 73-40-5; U, 66-22-8; C, 71-30-7; A, 73-24-5; f^5U, 51-21-8; D, 504-07-4; m^3U, 608-34-4; 3deazaU, 50607-28-8; tyrosyl-tRNA synthetase, 9023-45-4.

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